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Investigations of complement resistance factors in Leishmania chagasi

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in *Leishmania chagasi*

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Chapter 1

General Introduction

Leishmania are protozoan parasites that are the causative agents of a group of diseases collectively termed leishmaniasis, which range in severity of clinical manifestation from ulcerative skin lesions developing at the location of the insect bite, known as cutaneous leishmaniasis (CL) to disseminated, life threatening, visceral infection, known as visceral leishmaniasis (VL). These diverse clinical manifestations are determined by both the infecting species of *Leishmania* and the immune response of the host [1]. *Leishmania* parasites are transmitted to mammals, including humans, by the bite of phlebotomine sand flies and are capable of infecting nearly all vertebrates. They are unicellular, eukaryotic pathogens with a digenetic lifecycle, alternating between and replicating within an insect vector and a mammalian host. *Leishmania* exist within the sand fly vector as motile, extra-cellular promastigotes. In a mammalian host, *Leishmania* exist as nonmotile, spherical amastigotes that reside and proliferate within the phagolysosome of macrophages. When a sand fly ingests a blood meal from an infected host, the amastigotes are passed to the insect within ingested macrophages. As the macrophages lyse, the amastigotes are released into the midgut of the fly and quickly differentiate into the motile, flagellated form of the parasite called the procyclic promastigote. These promastigotes adhere to the midgut epithelium and are retained as the blood meal is digested and excreted. The procyclic promastigotes begin to divide and transform into additional promastigote forms, eventually leading to the generation of virulent, non-dividing metacyclics, capable of establishing infection in a successive host. The following work describes efforts to gain a more complete understanding of the process of metacyclogenesis, the process by which the infectious promastigotes are generated within the sand fly vector. Studies of infectious and culture modified parasites are used to identify and characterize factors involving one particular aspect of metacyclogenesis, resistance to lysis by complement factors in human serum.

Dissertation Organization

The objective of this dissertation was to identify and characterize factors involved with the parasite's ability to resist lysis by complement factors in human serum. The first study presents our approach to identifying target genes (contained within cosmid vectors) conferring resistance to complement mediated lysis (CML) in infectious promastigotes by employing genetic complementation to restore a CML-resistant phenotype to CML-sensitive parasite cultures. Several regions of genomic DNA were identified with the apparent ability to restore phenotype and a more detailed examination of one was undertaken through the complete sequencing of the identified cosmid. The second study details the further characterization of the genomic sequence contained within two of the CML-resistant cosmid constructs. This work details the sequencing of a second identified cosmid and the efforts to identify a discreet portion of cosmid insert sequence capable of conferring phenotype. Findings presented in the third data section of this thesis originate from an observation that was made independently of the original aim of the overall work. It was discovered that transfection with the cosmid shuttle vector used in our previous work in some way stabilizes the expression of RNA and protein specific for a virulence factor previously characterized in *leishmania*. This section details our efforts to confirm and begin to characterize this phenomenon. The fourth data chapter discusses our continuing efforts to identify a smaller region of the previously identified cosmid clone, capable of conferring CML-resistance to transfected cells. The appendix to this body of work contains descriptions of experiments that have contributed to the overall understanding of our biological system.

Literature Review

Epidemiology

There are 88 countries endemic for leishmaniasis with 12 million people currently infected and an additional 350 million people at risk of infection. There are 2 million new cases reported annually, with 500,000 of these being the potentially fatal, visceral form of disease [2] [3]. These figures are predicted to substantially underestimate the true global impact of leishmania infection due to under-reporting. Reporting is required in only 40 of the 88 affected countries [3] and many rural areas of affected regions have limited medical infrastructure. Factors contributing to a steady increase in the number of new infections occurring each year include urbanization, deforestation of rural areas, agricultural development and irrigation projects that result in increased contact between susceptible populations and parasitized insect vectors [4]. The incidence of Leishmania infection is also exacerbated by co-infection with a variety of opportunistic infections and HIV/AIDS [5] and emerging antileishmanial drug resistance [6].

Political instability and the suspension of government vector control programs, coupled with temporary or inadequate housing for groups of people forced to relocate due to conflict, natural disaster or economic hardship are resulting in localized epidemics of all clinical forms of leishmaniasis [7]. The upper Nile area of Sudan experienced a devastating epidemic of visceral leishmaniasis from 1984 to 1994 as drought, famine and civil war forced mass population movement and spread a variety of diseases among groups forced to live in conditions of malnutrition, inadequate shelter and poor sanitation. With nearly all medical facilities closed or without supplies, most cases of visceral leishmaniasis in the region were untreated; one retrospective study, based on verbal post-mortem, estimated 100,000 deaths due to leishmaniasis occurred in the population of 300,000 [8].

There are over 20 species of *Leishmania* that cause human leishmaniasis. New World *Leishmania* spp, including *L. braziliensis, L. amazonensis and L. mexicana* primarily cause cutaneous leishmaniasis (CL) and are most prevalent in South and Central American countries, as well as Middle-Eastern countries such as Afghanistan and Syria. Infection with three species of this group, *L. braziliensis, L. panamensis* and *L. amazonensis* can also result in secondary mucocutaneous leishmaniasis (MCL) within several years of resolving the original cutaneous disease. Old World *Leishmania* species such as *L. major* and *L. tropica* are responsible for cutaneous disease throughout Africa and Asia. Visceral Leishmaniasis (VL) is attributed primarily to the Old World species, *L. donovani and L. infantum* as well as the New World species *L. chagasi*. Visceral leishmaniasis can be found in Southern Europe, North Africa and Central and South America, with recent concentrated outbreaks in Sudan and India (reviewed in [6, 7, 9, 10]).

History

Anthropological evidence of cutaneous leishmaniasis can be found as early as the 15th century; both Incan text and the accounts of Spanish conquistadors mention the presence of skin lesions on agricultural workers from the Andes. However, the causative pathogen was not described until the turn of the 20th century. In 1900, Dr. William Leishman conducted an autopsy on a soldier who had died in England following a stay near Calcutta, India. While examining tissue samples from the spleen, he discovered small particles within macrophages which he called ovoid bodies. He published his findings in 1903, the same year that Dr. Charles Donovan described similar findings within splenic puncture specimens of living patients suffering from kala-azar. After finding the same distinct histological features in the two samples, the presence of both a nucleus and a smaller structure of mitochondrial DNA now know as the kinetoplast, these findings were determined to be the same organism and termed Leishman-Donovan bodies, with the species becoming known as *L. donovani*. Through the linkage of the parasite with the disease of kala-azar, Leishman and Donovan had discovered the genus of *Leishmania* (reviewed in *[10]*)[1]).

Parasite Development in the Sand Fly Vector

The *Leishmania* are protozoan parasites capable of infecting nearly all mammals. They are unicellular eukaryotic pathogens with a digenetic life cycle, involving alternation between a sand fly vector and a mammalian host. The cycle begins when a female sand fly vector takes a blood meal from an infected mammalian host; an event occurring on average every 4-5 days for most species of sand fly, that is required for the reproductive cycle of the vector [11]. The blood meal contains macrophages which harbor amastigotes, the intracellular form of the parasite. These amastigotes are spherical, non-motile and able to replicate within the acidic and hydrolase-rich phagolysosomes of the macrophage [12] [13, 14]. The ingested blood meal is rapidly encased within a non-lipid peritrophic membrane (PM), largely composed of chitin secreted by the epithelial cells of the sand fly gut. The formation of this membrane occurs during the intake of the blood meal. As macrophages are lysed, releasing amastigotes into the contents of the blood meal, the peritrophic membrane may protect the amastigotes from proteolytic enzymes secreted into the mid-gut in response to feeding [15]. These proteolytic enzymes reach maximum concentrations in the mid-gut 18-32 hours post feeding, which corresponds to the period of time (18-48 hours post feeding) in which amastigotes are transforming to the first of five flagellated forms of the parasite, the procyclic promastigote [16] (see figure 1).

Procyclic promastigotes are short (6-8μm), motile, replicative and are the prevailing form of parasites found within the peritrophic membrane at the point, 3 days post feeding, that the matrix begins to break apart. There is suggestive evidence that chitinases secreted by the procyclic promastigotes may be involved in initiating the breakdown of the PM, which occurs earlier than in blood fed, non-infected flies *in vitro* [17]. This chitinase activity may assist the parasites in establishing a stable infection in the fly. This mechanism of release from the PM may be at least partially responsible for the high degree of vector/parasite species specificity that exists in this system. It has been demonstrated that loss of infection in refractory vectors occurred following excretion of the digested blood meal and that the parasites in these flies were unable to escape the PM prior to excretion [18].

Even as the amastigotes continue to transform to procyclic promastigotes, procyclics begin to divide in the abdominal midgut. Within 2-3 days post feeding, a portion of the dividing procyclic population begins to transform to the long (12-20um), slender, highly motile but non-divisional form of the parasite termed the nectomonad (reviewed in [19]). There is a subset of the procyclic population that does not transform and continues to replicate. This population of persistent procyclics can be detected for the duration of infection and may serve as an ongoing supply of precursors to the other parasite forms in the fly (reviewed in [19]). As the motile nectomonads increase in concentration, they move forward in the fly gut and begin to accumulate and bind to the gut wall in the anterior portion of the abdominal midgut near the stomodeal (cardia) valve that controls the junction between the foregut and the midgut [20]. It has been demonstrated by electron microscopy that the nectomonads attach to the gut by inserting the flagella between the microvilli of the epithelium [21]. This attachment is mediated by specific binding of lipophosphoglycan (LPG) molecules on the surface of the parasite flagellum that recognize lectins on the surface of gut epithelium. The specificity of binding also contributes to the vector/parasite species specificity discussed above [12].

Within 3-7 days post feeding, a portion of the nectomonad population in the anterior abdominal midgut begins a further transformation to a second parasite form capable of replication, the leptomonad. Leptomonads are similar in length (6-8μm) to the initial divisional form, the promastigote. The appearance of this second divisional form represents a second growth phase in the development cycle of *Leishmania* in the fly vector [22]. As this population divides and accumulates in the lumen of the midgut, the leptomonads begin to secrete a filamentous proteophosphoglycan material called Promastigote Secretory Gel (PSG), which fills and distends the anterior midgut of the fly, eventually extending through the stomodeal value into the foregut [23]. This PSG plug contains a combination of embedded leptomonads and the infectious form of the parasite, the metacyclic promastigote.

Metacyclic promastigotes are short (5-8μm), motile, with a flagellum that is longer than the parasite body and are considered to be the infectious form of leishmania. Metacyclics are generated

from transforming leptomonads and can be detected as early as 5 days post feeding [22, 23]. This parasite form expresses a modified LPG molecule on its surface that prevents binding to the gut epithelium in preparation for inoculation into a host. The majority of metacyclics are associated with the leptomonads within the PSG plug, but they are also detectable in significant numbers at the poles of the plug, an ideal position for transmission during subsequent blood meals (reviewed in [20].

An additional parasite form detectable in mature infections within the fly vector is the haptomonad promastigote that appears concurrent with the leptomonad promastigote. The origin of these haptomonads is unknown, but based on timing and location it is likely that they are a further differentiated form of leptomonads [24]. The haptomonads attach to the cuticular lining of the stomodeal valve and the foregut. It is unclear what role this parasite form plays in the development and transmission of infection, but two possibilities have been suggested. The first proposes that they function as an early scaffold of cell bodies to facilitate the generation of the PSG plug (reviewed in [20]). The second theory proposes that haptomonads attach to the stomodeal valve, contributing to the mechanical blockage of the valve by secreting chitinases that destroy the chitin lining of the valve, thus abrogating its function of effectively regulating unidirectional flow of material from the foregut to the midgut [17, 25]. Despite the uncertain role of this promastigote form, they have been classified as an “altruistic” form that contributes to transmission of metacyclics, but cannot be transmitted themselves.

Transmission of infectious metacyclics during blood feeding

Once a mature infection is established in the fly, transmission is possible with the fly’s next blood meal and for all subsequent feedings that occur for the remainder of the vector’s lifespan. The number of infectious metacyclic promastigotes required to initiate an infection in a mammalian host varies by host and vector species, but is generally accepted to be in the range of 10-1000s [26, 27]. Natural fly feeding experiments have demonstrated that flies carrying mature *Leishmania* infections are not able to effectively engorge with a full blood meal, resulting in more frequent feeding on multiple hosts and repetitive probing during each attempted blood meal. Both behaviors are thought

to enhance parasite transmission [28]. These feeding difficulties have been attributed to a combination of the damage to the stomodeal valve, possibly from chitinases secreted by the attached haptomonad population, and the PSG plug that is secreted by the leptomonad population. The effect of these factors is the stomodeal valve is held open, facilitating a reflux of blood during feeding. This regurgitated blood has been shown to carry with it PSG from the plug, along with the associated metacyclic promastigotes that are embedded in the plug (reviewed in [19]). Because of the physical changes in the anatomy of the infected sand fly, the inoculum that is regurgitated back into the mammalian wound site contains infectious metacyclic promastigotes, PSG and sandfly saliva [27].

In order to successfully obtain a blood meal from a mammalian host, an insect must surmount the host's hemostatic system that includes the blood-coagulation cascade, vasoconstriction, fibrinolysis and platelet aggregation. These factors are influenced by the inoculation of sandfly saliva into the wound site during the initial stages of the blood meal [29, 30]. Sand fly saliva contains anticoagulants, anti-platelet factors, vasodilators, anti-inflammatory molecules and immunomodulating molecules (reviewed in [31]) and has been demonstrated to enhance *Leishmania* infectivity by modulating the local immune response to promote parasite survival, uptake by antigen presenting cells (APCs) and replication within APCs [11, 29, 30]. There is also significant evidence to suggest that hosts repeatedly exposed to uninfected sand fly bites develop antibodies against components of the inoculated saliva, which may play a role in host protection against future infection with *Leishmania* parasites[32]. Significant efforts are currently underway to employ components of saliva as protective vaccines in areas where leishmaniasis is endemic (reviewed in [30]).

Parasite Development in the host

Surface Molecules

Lipophosphoglycans

Lipophosphoglycan (LPG) is the most abundant surface molecule of promastigotes, covering nearly all surface area including the flagellum, it is also detectable at very low levels on amastigotes [33, 34]. LPG molecules have distinct domains including a glycosylphosphatidylinositol (GPI) lipid

anchor, a glycan core, phosphorylated oligosaccharide repeats (which are highly conserved among all *Leishmania* species) and an oligosaccharide cap [33, 35]. These molecules are produced by the parasite in the digestive tract of the sandfly as parasites differentiate from amastigotes into the various promastigote forms and LPG is differentially regulated during metacyclogenesis [36]. As procyclic promastigotes transform to infectious metacyclic promastigotes, the LPG molecules double in length by increasing the number of oligosaccharide repeats from an average of 14 to 30. Additional modifications in side chain composition and terminal capping of the molecule also occur during metacyclogenesis [35].

The stage specific nature of LPG modification suggests that it plays an important role in the development of infectious metacyclic promastigotes in the vector midgut. As previously discussed, the generation of a mature infection correlates with retention of procyclic promastigotes following digestion and excretion of the blood meal. Studies have implicated LPG as an adhesion molecule that mediates interaction of the promastigote with the midgut epithelium of the sandfly. *L. chagasi* promastigotes expressing procyclic LPG have been shown to bind to the vector midgut epithelium, but not promastigotes displaying metacyclic LPG. [37]. Additional studies with *L. major* promastigotes found that parasite binding to the vector midgut could be inhibited in a dose-dependent manner with the addition of purified LPG, and that LPG-deficient mutants were unable to bind and initiate infection [38]. These findings support the hypothesis that rearrangements in LPG structure of metacyclic promastigotes prevent binding of the parasite to the midgut and allow forward movement of the parasite to areas suitable for inoculation into a new host during subsequent blood feeding. The role of LPG in parasite virulence has been further characterized by the generation of LPG-deficient mutants. Mutants generated by targeted deletion of LPG1, which encodes a Golgi galactofuranosyltransferase and catalyzes an early step in GPI anchor synthesis, result in the specific loss of LPG without affecting related molecules such as secreted proteophosphoglycans [39]. These ΔLPG1 mutant promastigotes are significantly less virulent than wild type parasites for infecting isolated peritoneal macrophages and susceptible mouse strains [39, 40]. Conversely, ΔLPG1 amastigotes are equally

virulent to wild type amastigotes. Along with the finding that amastigote surface LPG is expressed very low levels in all *Leishmania* species, these findings support the hypothesis that LPG is important for promastigote survival in the vector, but not essential for amastigote survival in the host [34, 39].

Major Surface Protease

Major surface protease (MSP) is the most abundant glycoprotein on the promastigote cell surface and the second most abundant surface molecule. In the literature, this molecule is referred to by several designations including MSP, which reflects the location of the protein, GP63 to reflect the size (63 kD) and glycosylation state, and leishmanolysin to indicate the protease activity of the protein (reviewed in [41]). Mature, fully processed MSP acts as a zinc metalloprotease. The immature protein's N-terminal domain contains a pro-peptide with a cysteine residue bound to a zinc atom at the active site to inhibit enzyme activity. This pro-peptide is cleaved during maturation to render an activated product, which is transported for GPI-anchoring to the cell surface [42, 43].

MSP coding sequences are highly conserved among *Leishmania* species [44] and have been identified in other trypanosome parasites. The African trypanosome *Trypanosoma brucei*, the causative agent of African sleeping sickness, was found to have at least three gene families encoding homologues of *Leishmania* MSP [45]. In *Leishmania chagasi*, MSPs are encoded by tandem arrangements of gene clusters and are differentially expressed as three separate classes of mRNA during *in vitro* culture of promastigotes. These classes are *MSP*-L encoding a 2.7kb mRNA expressed predominantly in logarithmic phase promastigotes, *MSP*-S encoding a 3.0kb mRNA expressed in stationary phase promastigotes and *MSP*-C encoding two mRNA species of 2.3kb and 3.1kb expressed constitutively throughout *in vitro* promastigote growth. [46-48]. The major divergence among the three mRNA species occurs in the sequence and length of their 3' untranslated regions (UTRs) and the downstream intergenic regions (IRs) [48]. The 3'UTR regions were implicated in the differential regulation of mRNA levels with experiments that fused a reporter gene to the three *MSP*

UTRs and resulted in regulation of the reporter with similar patterns to the corresponding *MSP* [47, 49, 50].

MSP is detectable on the surface of both promastigotes and amastigotes. Logarithmic promastigotes express a single 63kDa isoform of MSP on their surface while promastigotes in the stationary phase of growth express both the 63kDa and a 59kDa form on their surface. Neither of these isoforms are detectable on the surface of amastigotes, being replaced by a 64kDa form [51, 52]. These protein isoforms likely serve different functions in the various stages of parasite development. MSP has been shown to bind C3 proteins of the complement cascade, an important component of the mammalian innate immune response [53]. Following binding, the endopeptidase activity of MSP is able to cleave C3 to the breakdown products C3a and C3b [54]. Normal activation of the complement cascade also results in the generation of C3b, which can amplify additional C3b deposition by cleaving C3 proteins to yield an active C5 convertase. MSP has been implicated in disrupting the complement cascade through the enzymatic conversion of C3b to the inactive iC3b. Studies found that expression of MSP on the surface of Chinese Hamster Ovary (CHO) cells, upon exposure to serum, leads to a conversion of C3b complement factor to an inactive form [55]. Such a cleavage event would abolish the activation of downstream complement components and formation of the membrane attack complex (MAC). Inactivated iC3b remains associated with the parasite membrane and may function as an opsonin, resulting in receptor mediated phagocytosis of the promastigote by host macrophages. Macrophage receptor CR3 binds iC3b on the promastigote surface [56, 57] and receptor CR1 binds both C3b and iC3b [58].

Targeted gene replacement was used to generate *L. major* promastigotes lacking all seven MSP genes [59]. The resulting promastigotes lacked MSP expression at all developmental phases. These mutants showed normal patterns of development within the appropriate sand fly vector, and were capable of infecting mouse macrophage cells and differentiating to the intracellular amastigote form. The mutants were found to cause a delay in lesion formation when used to infect BALB/c mice,

suggesting a decrease in parasite virulence. Mutant promastigotes were also found to have a significant increase in sensitivity to complement-mediated lysis (CML) when exposed to human serum. These deficiencies were restored with the restored expression of a cloned MSP gene [59]. These findings strongly support the role of MSP as an important virulence factor in *Leishmania.*

Promastigote Surface Antigen

Promastigote Surface Antigen (PSA) is an abundant protein expressed on the surface of infectious promastigotes in all *Leishmania* sp. examined except for those belonging to the *Leishmania braziliensis* group [60, 61]. As a 46-kDa glycoprotein, this molecule is also commonly referred to as GP46. Levels of PSA mRNA and expression of GPI-anchored PSA have been shown to increase 11-30 fold as promastigotes transition from a logarithmic to stationary phase of growth [62]. This differential regulation of mRNA and protein hints at the possible role of this molecule as a virulence factor, correlating with multiple characteristics of metacyclogenesis. These include the progression of the parasite from a procyclic promastigote with low infectivity in logarithmic growth phase to a metacyclic promastigote with high infectivity in stationary growth phase, and the increase in mRNA and protein expression levels of MSP, an active virulence factor in metacyclic promastigotes. Interestingly, PSA expression, MSP expression and parasite virulence to a mammalian host are lost as promastigotes are serially passaged in culture media over 15 weeks [62-64].

Although there is limited understanding of the mechanism by which PSA contributes to the success of the parasite glycocalyx in protecting the metacyclic promastigote from the host immune system and aiding in the establishment of infection, there are two features of the protein structure that may prove interesting during additional characterization of the molecules function. The first is the presence of multiple leucine rich repeat regions (LRRs) consisting of 24-26 amino acid residues in the amino-terminal region of PSA [62]. LRR motifs are known to be involved with protein-protein interactions important in signal transduction, cell adhesion, DNA repair, recombination, transcription and RNA processing as well as pathogen recognition and host cell invasion [65].The LRR motif of

PSA is also found in secreted proteophosphoglycan molecules (PPG) [66] that have been shown to bind macrophages and are internalized into the same lysosomal compartments to which phagocytosed promastigotes are targeted [67]. Native and recombinant PSA-2 was found to be involved in binding bind murine macrophage receptor CR3 as well as the CR3 receptor of a human monocytic cell line, indicating that PSA LRRs may function to mediate parasite invasion [68]. The other structural feature of interest of PSA is located near the carboxyl-terminal membrane anchor, where a Thr/Ser-rich region may serve as a potential site for *O*-linked post-translational modifications. A similar motif is present in human decay activating factor (DAF), a glycolipid-anchored membrane protein involved in complement resistance that functions by inhibiting the formation and accelerating the decay of C3/C5 convertase complexes of the human complement cascade [69].

Based on the similarity in location and expression patterns to MSP, a known CML-resistance factor in *Leishmania,* and sequence similarity to DAF, it was proposed that PSA may play a role in resistance to CML in infectious *Leishmania* promastigotes. While a mechanism for this has not been determined, one initial study examined the effects of expressing *PSA* on an episomal plasmid in CML-sensitive, serially passaged promastigotes lacking expression of PSA and MSP. The resulting transformed promastigotes were found to express PSA protein in the stationary growth phase and to have significant levels of restored CML-resistance [64]. Further studies are necessary to determine a mechanism for this observed phenotype, but it appears likely that PSA is contributing to the infectious metacyclic promastigotes ability to circumvent the host immune response and establish infection.

Evasion of Complement

Once introduced into the blood pool created by the feeding sand fly, metacyclic promastigotes immediately encounter the first line of defense in the mammalian innate immune system, the lytic serum factors of the complement cascade. The complement cascade is comprised of a group of serum proteins that sequentially bind to the surface of a pathogen to activate the immune

system and lyse the invading cell by insertion of a membrane attack complex (MAC) into the cell surface. The complement cascade can be activated by any of three pathways. The classical pathway is activated when circulating antibodies bind to the surface of the pathogen, the alternative pathway is activated by direct binding of a complement protein (C3) to the pathogen surface, and the lectin binding pathway is activated when lectin protein recognize molecular patterns on the pathogen surface (reviewed in [70]). Although the activating molecules are different for each, the three pathways converge at one point by activating a serine protease to form the C3 convertase that cleaves the central C3 component into 2 subunits, C3a that is released to act as an inflammatory mediator and C3b that binds the pathogen surface. Any C3b molecules that are released into the bloodstream are rapidly inactivated by hydrolysis, while bound C3b can bind additional complement factors (B and D) and become an additional active C3 convertase, accelerating the generation and binding of C3b to the cell surface [70].

There are two potential outcomes for the parasite. The first outcome involves a successful immune response that results in parasite lysis and clearance. This occurs when the bound C3b complexes to additional complement proteins to form a C5 convertase. This enzyme complex facilitates formation of an anchor-like structure on the surface of the pathogen that binds between 10-19 molecules of C9. The C9 molecules in turn polymerize to form a ring structure called the Membrane Attack Complex (MAC). This ring structure possesses a hydrophilic external face which allows it to insert into the lipid bilayer and form a hydrophobic internal channel. The successful disruption by the MAC results in cell lysis due to the loss of membrane potential and cellular homeostasis, as well as the penetration of destructive enzymes such as lysozyme and nucleases [71]

The second possible outcome involves parasite evasion of MAC lysis and subsequent phagocytosis by Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. It has been demonstrated in all *Leishmania* spp. examined that metacyclic promastigotes are resistant to complement mediated lysis (CML), while procyclic promastigotes are CML-sensitive [72-76]. This increase in CML-resistance correlates with other characteristics of metacyclogenesis such as

increased infectivity to mammals, up-regulated expression of MSP and PSA and lengthening and modification of LPG molecules on the promastigote surface.

Metacyclic promastigotes utilize opsonization with complement components to facilitate receptor-mediated uptake by macrophages [12, 58, 77]. Parasite entry into macrophages has been demonstrated to be a passive process with studies showing that pre-treatment with cytochalasin B, an inhibitor of phagocytosis, prevents uptake of bound parasites and that heat killed promastigotes were still phagocytosed by macrophages [78]. As discussed earlier, these parasites are able to survive exposure to complement proteins by a variety of interactions with the parasite membrane anchored proteins. It has been demonstrated that MSP and LPG, the two most abundant surface molecules on this stage of promastigote are also C3 acceptor sites [53, 54]. MSP, a zinc protease, rapidly converts C3 to C3b and then further to the inactive form iC3b. This prevents binding of downstream complement components and MAC formation, thus protecting the parasite from CML [55]. LPG molecules on the surface of metacyclic promastigotes increase to approximately twice the length of the LPG on procyclic promastigotes and protect metacyclics from CML by positioning functional MAC complexes formed on the LPG molecules at a location too distal to penetrate the parasite membrane and cause cell lysis [35]. PSA has also been implicated in CML-resistance, potentially through a structural contribution to the dense glycocalyx on the surface of metacyclic promastigotes, but a specific mechanism has not been established for this glycoprotein [64].

Once a metacyclic promastigote attaches to the surface of a mammalian macrophage through receptor mediated binding, it is endocytosed into a phagosome known as the parasitophorous vacuole (PV), which fuses with a lysosome vacuole to become a lytic phagolysosome [12]. The promastigote is immediately introduced to an acidic and hydrolase-rich environment within the PV. This acts as a trigger for the promastigotes to begin transformation to the intracellular amastigote form of the parasite that is adapted for survival and replication within the vacuole. During the initial stages of transformation, LPG on the promastigotes surface protects the

parasite from the harsh environment of the vacuole and transiently inhibits phagosome maturation to allow sufficient time for promastigotes to differentiate into hydrolase-resistant amastigotes [12, 79].

Once completely differentiated, amastigotes are able to further modulate the host immune response, allowing them to survive and multiply within the PV [80]. Following amastigote replication, amastigotes are released from the macrophage and are able to infect other local macrophages. Although the mechanism of amastigote release is yet unknown, the current accepted view is that the infected macrophages burst and the amastigotes spill out. Recent video-microscopy data suggests that vacuoles containing amastigotes accumulate at the periphery of the infected macrophages, releasing amastigotes over several hours in a process similar to exocytosis [81]. Released amastigotes are the disseminating parasite form in mammals and spread to additional APCs in the mammalian system. These intra-cellular parasites are the source of infection for a sandfly taking a blood meal from an infected host.

Kinetoplast

Leishmania are among a group of protozoa that contain both nuclear DNA and a separate structure of mitochondrial DNA known as kinetoplast DNA (kDNA). The structure of the kinetoplast DNA is a network comprised of rings, thousands of minicircles and dozens of maxicircles interlocked together to form concatemers that result in a condensed, disk-shaped structure [82]. The products of this unusual DNA organization are involved in regulation and the generation of rRNA products, similar to mitochondrial DNA from higher eukaryotes. Successful generation of these transcripts requires both the maxi and minicircle DNA as the maxicircle transcripts are cryptic and require RNA editing to form a functional transcript. This editing involves insertion or deletion of uridylate residues at very specific internal sites of the maxicircle transcript, with specificity of editing controlled by minicircle-encoded guide RNAs.

Leishmania Genome

In 2005, the sequencing project for the 32.8-megabase genome of *Leishmania major* (Friedlin strain), the chosen reference strain, was completed and published [83]. This project provided sequence data for the 36 chromosomes of the haploid genome and predicted for 911 RNA genes, 39 pseudogenes and 8272 protein-coding genes [83]. This information allowed for extensive microarray analysis that revealed that over 90% of identified genes have significant levels of expression and that the majority of these genes are expressed constitutively in all developmental stages, with 0.2%-5% differentially expressed (reviewed in [84]). Although the size of the chromosomes within the genome vary (0.3Mb to 2.5Mb) [85], the organization of genes within the chromosomes is similar.

Leishmania genes are organized as large clusters of hundreds of genes, arranged in a head to tail orientation. Chromosome 1 is a useful example as it contains a 257kb region that is densely packed with 79 protein-coding genes. This region is flanked by telomeric repetitive elements and contains a 1.6kb stretch lacking open reading frames within the gene dense portion (see figure 2). This region is commonly referred to as the strand-switch region, with 29 predicted open reading frames (pORFs) located on one strand that are oriented toward the left telomere, and 50 pORFs on the opposite strand, oriented towards the right telomere [85, 86]. RNA transcription occurs by RNA polymerase II, but initiation occurs in the absence of detectable type II promoters (reviewed in [87]). Transcription initiates at the strand-switch region located at the 5' end of each gene cluster and proceeds in a polycistronic manner toward the telomere region. The lack of individual pol II promoters results in all pORFs on the strand being constitutively transcribed [88] with genes that require higher levels of expression being arranged in tandem arrays of identical genes, thereby resulting in multiple copies of mRNA [83].

Genes clustered in common orientation and transcribed on the same polycistronic RNA transcript often do not share common function or regulation and are cleaved into individual mRNAs through a trans splicing reaction. The absence of introns within pORFs results in complete splicing of

mRNA transcripts occurring via two coupled events. The first event is the insertion of 39 nucleotide cap called the splice leader into a splice acceptor site upstream of the 5' end of the ORF [89]. There are between 100-200 splice leader genes in the *Leishmania* genome, depending on species, which are arranged in tandem clusters in a head-to-tail alignment. These transcripts account for ~6% of RNA synthesis within the parasite (reviewed in [90]). Splice leader genes are transcribed by RNA polymerase II, initiated by an upstream promoter sequence and terminated by a downstream set of 5-31 thymidine residues [91]. The second event that is necessary for complete trans-splicing of the polycistronic mRNA transcripts is polyadenylation, which is driven by the insertion of the splice leader for the downstream ORF [89]. Polyadenylation occurs at a fixed distance (400-500 nucleotides) from the downstream splice leader insertion site. Experiments that altered the position of a downstream splice acceptor site resulted in a shift of the upstream poly(A) site to a location 400-500 nucleotides from the new splice acceptor. Related experiments eliminated the upstream poly(A) site and found no change in the location of the downstream splice acceptor site, indicating that the fixed distance between the two splicing events is determined by the location of the splice acceptor [92].

As *Leishmania* lack normal eukaryotic transcriptional control of gene expression, stage-specific regulation of expression occurs via post-transcriptional mechanisms. The majority of post-transcriptional regulation identified in *Leishmania* to date implicates involvement of the 3' untranslated region (UTR) corresponding to the regulated gene. Multiple studies have demonstrated the ability of the 3' UTR region to regulate a reporter gene in a similar pattern to the differentially regulated *Leishmania* gene. There are multiple examples of 3' UTR elements regulating protein expression through the modulation of RNA stability and abundance [41, 48, 62, 93], as well as increased translation in the absence of increased mRNA stability [94, 95]. The regulation of genes and corresponding proteins in *Leishmania* is of great interest to those seeking to understand mechanisms of virulence and host immune evasion. As the parasites transition between insect vectors and mammalian hosts, they encounter vastly different environments and challenges to survival. The

constitutive nature of transcription may play a vital role in their ability to quickly adapt to this dynamic life cycle.

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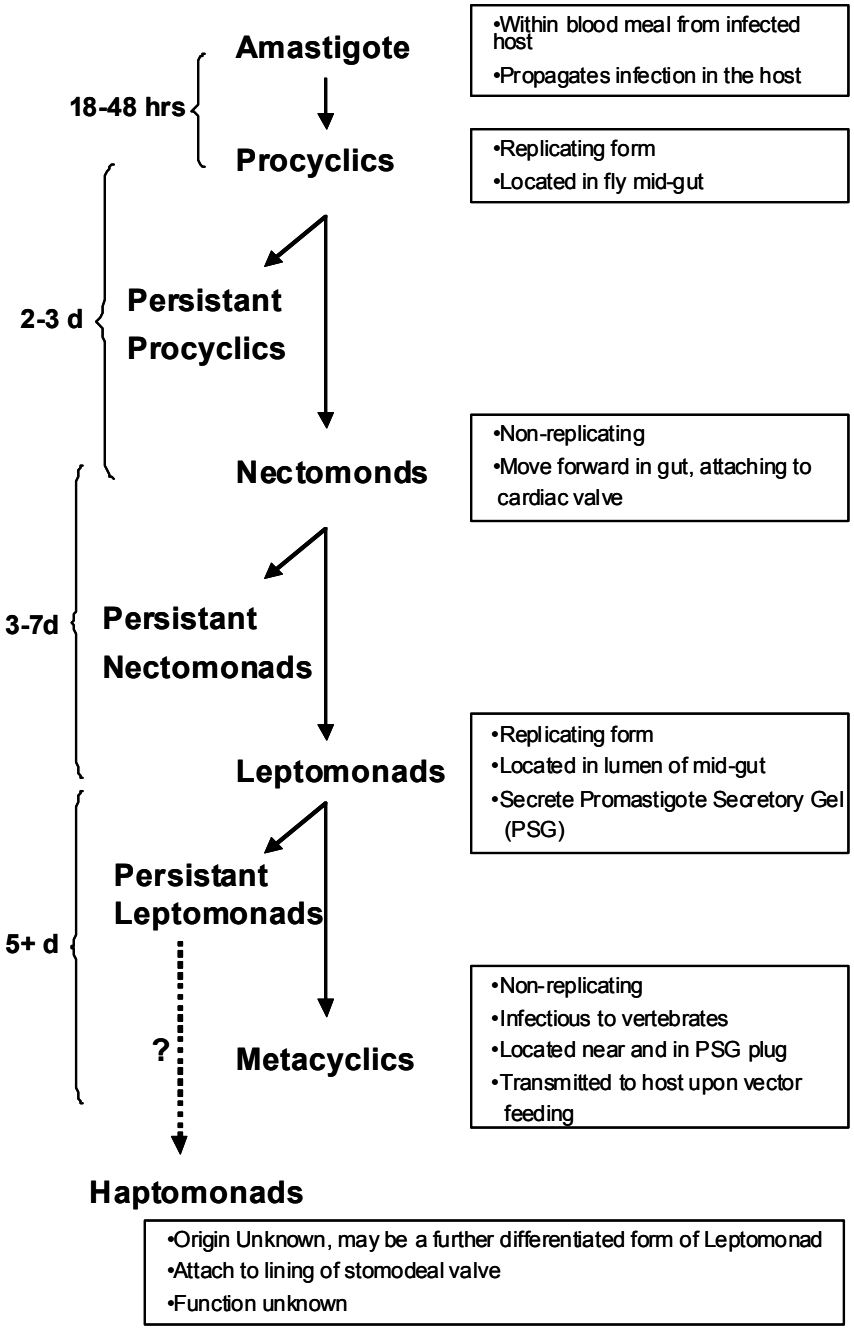

Figure 1. Promastigote forms identified in the sand fly vector.

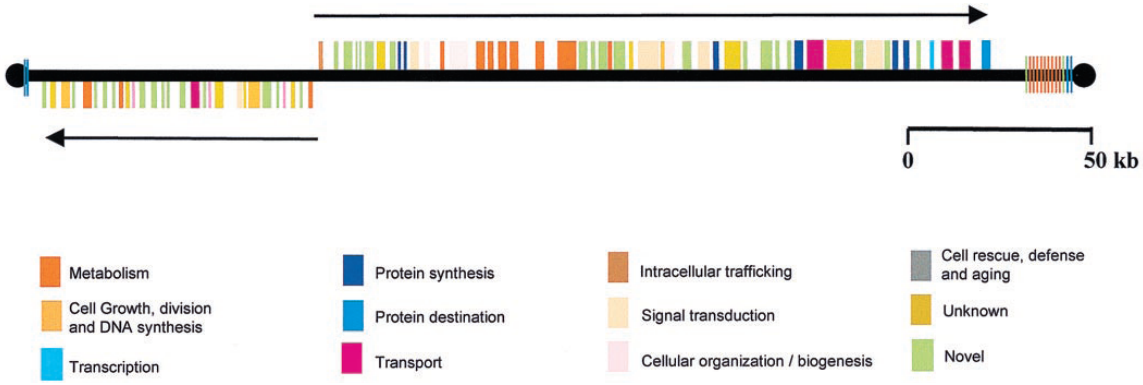

Figure 2.
Relative locations of the 79 genes in the smaller of the two homologues of chromosome 1 in *L. major*. The thick black line and black circles indicate the DNA and telomeres, respectively. The boxes depict the genes and are color-coded according to the potential functions of their protein products. The ''unknown'' genes have sequence similarities to genes of unknown function in other organisms. The ''novel'' genes have no substantive similarities with other sequences in the databases. The 50 contiguous genes on the top strand, including 5 tandem genes encoding long-chain fatty acyl CoA synthetases, are transcribed to the right (3); the 29 contiguous genes on the bottom strand are transcribed to the left (4). The colored vertical lines adjacent to the telomeres indicate telomeric and subtelomeric repeats. The larger of the two chromosome 1 homologues (not shown) has an identical gene organization, but contains an additional 201 kb of subtelomeric repeats at the right telomere (Donelson, J.E., PNAS 96(6), 1999).

Chapter 2

Genetic complementation to identify DNA elements that influence complement resistance in *Leishmania chagasi*

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Abstract

Past studies showed that *Leishmania spp.* promastigotes exhibit differential sensitivity to complement mediated lysis (CML) during development in vitro and in vivo. *Leishmania chagasi* promastigotes in cultures during logarithmic and stationary growth phases are CML-sensitive or – resistant when exposed to human serum, respectively, but only in cultures recently initiated with parasites from infected animals; serially passaged cultures become constitutively CML-sensitive regardless of growth phase. Building on these observations, a genetic screen was conducted to identify novel complement resistance factors of *L. chagasi*. A cosmid library containing genomic DNA was transfected into a promastigote line previously subjected to >50 serial passages. Selection with human serum for CML-resistance yielded 12 transfectant clones. Cosmids isolated from 7 of these clones conferred CML- resistance when transfected into an independent high passage promastigote culture; at 12% human serum, the mean survival of transfectants was 37% (± 11.6%), and of control transfectants was about 1%. Inserts within the 7 cosmids were unique. Determination of the complete DNA sequence for 1 cosmid indicated that its 32 kb insert was 89% identical (overall) to a 31 kb region of *Leishmania major* chromosome 36 that is predicted to encode 6 genes, all of which encode hypothetical proteins.

Introduction

Leishmania spp. (Trypanosomatidae) are the etiologic agents of leishmaniasis, a disease group that ranges in severity from self-healing cutaneous lesions to potentially fatal visceral infections. These protozoan parasites alternate between an intracellular amastigote form located within phagocytic cells of the vertebrate host immune system and a promastigote form located in the midgut of the sandfly vector. After a female fly bloodfeeds on an infected host, amastigotes within ingested phagocytic cells are released and quickly differentiate into procyclic promastigotes that attach to the midgut wall. Over a period of 1 to several weeks within the fly, promastigote development is characterized by a progression through a series of intermediate parasite stages that are distinguishable by location, morphology and replicative/non-replicative status (Rogers et al., 2002). The progression yields the final stage, the metacyclic promastigote. Metacyclic promastigotes migrate to the anterior gut, where they become available for inoculation into a mammalian host when the sandfly next takes a bloodmeal (reviewed in Schlein, 1993). Metacyclic promastigotes are distinguishable from procyclic promastigotes based on their morphology, high infectivity to vertebrates (Sacks and Perkins, 1984; Schlein, 1993; Saraiva et al., 1995), increased or differential outer surface glycosylation state, including of lipophosphoglycan, or LPG, (Sacks et al.,1985; Sacks et al., 1995), and increased expression of surface glycoproteins major surface protease (MSP, also known as GP63, (Wilson et al., 1993)) and promastigote surface antigen (PSA, also known as GP46 (Beetham et al., 1997)). Importantly, these properties of procyclic and metacyclic promastigotes are mirrored when parasites recently isolated from parasitized vertebrate hosts are grown in axenic cultures as promastigotes; procyclic and metacyclic promastigotes are found in cultures during logarithmic and stationary growth phases, respectively (Sacks and Perkins, 1984; Zarley et al., 1991).

Another trait of metacyclic promastigotes of all *Leishmania* spp. examined is resistance to the complement component of the vertebrate immune system (Franke et al., 1985; Joshi et al., 1998; Noronha et al., 1998; Pinto-da-Silva et al., 2002). The host complement system is one of the first antimicrobial immune mechanisms encountered by newly inoculated metacyclic promastigotes. Complement consists of multiple serum proteins having the potential to bind in series to invading

microbial pathogens. Complement recognition/binding can kill microbes by either of two mechanisms. In one, complement proteins bound to microbes function as opsonins that facilitate receptor-mediated uptake by phagocytic cells. In the other, completion of the complement cascade results in formation of a membrane attack complex on the microbial cell surface that induces cell lysis by disrupting the membrane integrity, a process called complement mediated lysis (CML).

Interestingly, metacyclic promastigotes utilize complement opsonization to facilitate their receptor-mediated entry into phagocytic cells within which the parasites survive and replicate during their amastigote life stage (Blackwell et al.,1985; Mosser and Edelson, 1985; Da Silva et al., 1989; Puentes et al., 1990; Dominguez and Torano, 1999). To effectively use complement towards gaining entry into phagocytes, metacyclic promastigotes must also avoid CML. Past studies have shown that metacyclic promastigotes do avoid CML, and that this property may involve three highly abundant surface macromolecules, MSP, LPG, and PSA. MSP, a zinc protease, is believed to block the complement cascade by MSP-induced proteolysis of complement factor C3b to an iC3b-like form that retains opsonin function while losing additional activities required for assembly of the membrane attack complex (Brittingham et al., 1995). LPG-induced protection to CML is associated with a lengthening of LPG and/or a change in terminal glycosylation residues, and these changes may act by preventing proper localization of membrane attack complex, relative to the parasite surface membrane (Puentes et al., 1990). Restored expression of PSA in PSA-minus high passage promastigotes resulted in increased resistance to serum lysis (Lincoln et al., 2004); the mechanism for this effect has not been determined.

Pathogens have evolved multiple mechanisms with which to evade the antimicrobial effects of complement-activation, and frequently individual species (including, as noted above, of *Leishmania* spp.) have several complement-evasion strategies that can operate simultaneously and independently (Wurzner, 1999). One approach that, to our knowledge, has not previously been used to identify novel molecules involved in microbial resistance to CML is that of genetic

complementation. Recent studies determined that serial passage of *Leishmania chagasi* causes promastigotes to become constitutively sensitive to CML regardless of culture growth state (Lincoln et al., 2004). Experiments were, therefore, undertaken that aimed, by genetic complementation of serially passaged cells, to identify genetic elements that function in CML-resistance in *L. chagasi*.

Materials and Methods

Parasites

Infectious *L. chagasi* amastigotes (strain MHOM/BR/00/1669, originally isolated in Brazil from a patient with visceral leishmaniasis) were maintained in Golden Syrian hamsters by serial passage as described (Pearson and Steigbigel, 1980). Amastigotes were differentiated into promastigotes, and the promastigotes were subsequently cultured, using supplemented Modified minimum essential media (HOMEM) as previously described (Ramamoorthy et al., 1992). Promastigote cultures seeded at 1.0×10^6 cells/ml were split to 1.0×10^6 cells/ml seven days later, a time that corresponded to 2-3 days after reaching stationary growth phase with a density of $2\text{-}4 \times 10^7$ cells/ml. Logarithmic and stationary phases of cultures were determined by cell morphology and culture density as described (Zarley et al., 1991). Cells cultures were considered low passage (LP) if serially passaged for ≤3 wk. High passage cells (HP) used in the following experiments were passaged for >50 wk.

Human Serum

Human serum was derived from the pooled extracts of multiple donors. Blood drawn from naive human donors into serum separator tubes was incubated for 1 hr at 23 C, then 1.5 hr at 0 C and then centrifuged at 450 g for 10 min at 4 C. Resulting serum fractions were pooled, aliquoted, stored at -80 C, and thawed on ice when needed for assays.

Complement Assays

Promastigotes were pelleted by centrifugation at 1,000 g for 5 min., washed in phosphate buffered saline (PBS, pH 7.8), then resuspended in PBS at 7 x 10^7 cells/ml. Cell aliquots (50 µl) in 96-well plates were brought to 100 µl total volume with PBS and/or human serum, then incubated at 37 C for 30 min. Cells were then diluted 1:10 in 0 C PBS and motile cells were counted on a hemocytometer. At high serum concentrations, non-viable, CML-sensitive cells are seen mainly as cell debris, while at lower serum concentrations CML-sensitive cells often are still visible but appear as non-motile and granular "ghost cells" that were not counted as viable. The percentage of cell survival was calculated as the ratio of motile cells present in incubations with, versus without, serum.

Library construction

Total genomic DNA isolated by kit (DNAzol, Invitrogen, Carlsbad, California) from 2-3 x 10^9 low passage promastigotes was partially digested with restriction endonuclease *Sau*3A to yield DNA fragments of predominantly 20-40 kb (as determined by ethidium bromide staining of electrohporetically separated digest products). Digested DNA was ligated into *Bam* HI –digested cosmid vector cLHYG (a kind gift from S. Beverley) then packaged into viral particles and amplified in *Escherichia coli* by kit (Gigapack® III XL, Stratagene, La Jolla, California). Phage-infected bacterial colonies were pooled (3 pools, ~2,700 colonies per pool), individual pools were amplified in *E. coli*, and then cosmid DNA was isolated (Maxi Kit, Qiagen, Valencia, California) for use in parasite transformation experiments.

Transfection and selection of parasites

Transfection of high passage promastigotes by electroporation of cosmid DNA was largely as described (Kapler et al. 1990), excepting that electroporation was in 75% electroporation buffer (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and 25% CytoMix (120 mM KCl 0.15 mM $CaCl_2$, 10 mM K_2HPo_4, 25 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$). Electroporation in 0.4 cm cuvettes used the following electrical constraints: 2 kV and 25 µfd. High

passage promastigote cultures in late logarithmic growth phase (0.8-1.0 x 10^7 cells/ml) were transfected using 8x10^7 cells per transfection reaction. Following transfection, cells were incubated 72 hr in 1X M199 media (Cellgro, Herndon, Virginia) to allow recovery. Cells were pelleted by centrifugation for 5 min at 1,000 g, resuspended in HOMEM containing 25% pooled human serum (see human serum), and incubated at 37 C for 30 min. Cells were again pelleted, the pellet washed in PBS and resuspended in 500 µl HOMEM and spread onto semi-solid media containing M199 medium (Invitrogen) supplemented with 100 µg/ml hygromycin (Sigma, St. Louis, Missouri). Colonies were isolated from plates 2-3 wk later, and then individual isolates were cultured in HOMEM supplemented with 10% heat inactivated-fetal calf serum and 100 µg/ml hygromycin.

Cosmid DNA was recovered from transfected promastigotes using a modified alkaline lysis procedure. Promastigotes from stationary phase cultures (2 x 10^8 cells) were pelleted at 1,500 g for 5 min, resuspended in TGE buffer (25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose), and lysed in 200 µl 0.2 M NaOH:1% SDS. Following 5 min incubation on ice, the protein was precipitated by adding150 µl Na acetate, pH 4.8, then centrifuging at 14,000 g for 5 min. The resulting supernatant was extracted (with phenol:chloroform:isoamyl alcohol, 25:24:1), and the DNA within the aqueous phase was precipitated by the addition of 2 volumes ethanol and centrifugation (5 min, 14,000 g). Precipitated DNA was resuspended in 200 µl buffer (25 mM Tris pH 8.0, 10 mM EDTA pH 8.0), then re-precipitated by bringing the solution to 6.5% in polyethylene glycol (weight by volume) and 0.4 M in NaCl, incubated on ice for 1 hr and pelleted by centrifugation (5 min, 14,000 g). The resulting pellet was washed (in 70% ethanol) then resuspended in 20 µl buffer (25 mM Tris pH 8.0, 10 mM EDTA pH 8.0).

Sequencing and annotation

To sequence the entire insert of cosmid #2, cosmid DNA fragments resulting from *Sac* I digestion were cloned into the *Sac* I site of plasmid *pBluescript* SK(-) (Stratagene), or were subjected to re-ligation. Initial sequencing reactions used universal primers that annealed to the plasmid

sequence flanking the inserts; subsequent reactions used primers that annealed to sequence that had been determined in the prior sequencing reaction. Sequences from the subclones were aligned and connected by directly sequencing cosmid template using "outward-facing" primers designed against the sequence of the ends of the subclone inserts. Sequencing of the ends of the inserts within cosmids #1 and #3-6 used primers that annealed to cosmid vector sequence flanking the inserts. Cosmid insert sequences were analyzed for predicted coding sequences and for similarity to the *L. major* genome (at http://www.genedb.org, Sanger Inst. U.K.) using several software programs: GLIMMER and Artemis (with the assistance of Al Ivans, The Sanger Institute, Cambridge, U.K.), BLAST, GAP and BestFit.

Results

Only *L. chagasi* parasites within low passage stationary phase cultures resist CML.

Complement sensitivity assays using human serum were performed on promastigotes taken from low and high passage cultures at logarithmic and stationary growth phases. High passage cells were CML-sensitive in cultures during both logarithmic and stationary growth phases (LC50= 4.7% and 4.8% serum, respectively; Fig. 1b). In comparison, low passage cells exhibited a differential CML-sensitivity that was dependant on the culture growth phase (Fig.1a). Cells from low passage cultures during logarithmic growth phase were highly sensitive to CML (LC50=4.5% serum), while cells from stationary phase cultures were highly resistant, with nearly 100% surviving even at the highest serum concentrations; consequently, the LC50 could not be calculated/extrapolated for these CML-resistant cells.

To screen for genetic elements able to influence CML-sensitivity in promastigotes from high passage cultures, a genomic complementation library was constructed using the cosmid vector cLHYG; DNA elements contained within this vector allow cosmid replication and drug resistance selection in *Leishmania* spp. and in *E. coli* (Ryan et al., 1993). Partial restriction enzyme digestion (using *Sau*3A) of *L. chagasi* genomic DNA yielded 20-50 kb DNA fragments that were ligated into

cosmid cLHYG. Following transfection of *E. coli* with packaged cosmid, the insert size of 20 randomly selected bacterial cosmid clones was determined to range between 22 to 40 kb (mean=35 kb).

Identification of 6 cosmid-transfected high passage clones having increased resistance to CML.

To obtain a heterogeneous pool of cosmids, bacterial colonies containing the library clones were pooled (3 pools, each with 2,700 colonies per pool), amplified, and then their cosmid DNA was isolated. The resulting pooled cosmid DNA, representative of 8,700 colonies, was transfected by electroporation into CML-sensitive, high passage promastigotes from the same culture line as was used for the CML-assays reported in Figure 1. To select for CML-resistant parasites, promastigotes from high passage cultures in late logarithmic growth phase were transfected by electroporation (see Methods) and allowed to recover in 1 x M199 for 48 hr. Transfectants were concentrated by centrifugation and resuspended in PBS containing 25% naive human serum for 30 min. Surviving transfectants were clonally isolated on semi-solid medium and then expanded in liquid medium (see methods). Assay of the clonal isolates for CML sensitivity identified 20 clones that were CML-resistant, of which 12 were selected for additional analysis.

Restored CML-resistance is associated with the cosmid construct.

Based on their CML-resistant phenotype, clones were analyzed to confirm that the CML-resistance was due to cosmid insert DNA. Initially, cosmids were successfully recovered/isolated from 8 of 12 CML-resistant clones (see methods). Two of these 8 cosmids were determined to contain identical inserts (in experiments described later within this paper); subsequent experiments described herein pertain to the remaining 7 cosmids only. The 7 purified cosmids were then transfected back into CML-sensitive promastigotes, i.e., promastigotes from high passage cultures, and resulting transfectants were clonally isolated and assayed for CML-sensitivity. In all cases, the CML-resistant phenotype “followed” the cosmid constructs; each transfectant clone exhibited CML-

resistance when exposed to 12.5% serum. The survival of individual clones ranged from means of 22% to 54%, and the combined average survival among all clones was 37% ± 11.6% at 99% confidence interval (Fig. 2). The CML-resistance of these clones was not due to the cLHYG backbone within the cosmid constructs; control transfectants containing cLHYG with an irrelevant insert were as CML-sensitive as the non-transfected, high passage cells, i.e., column HP-C (Fig. 2). The positive control included in all experiments (low passage promastigotes from stationary phase cultures) always yielded survival rates of nearly 100% at all serum concentrations tested. When the 6 clonal transfectants were assayed at higher serum concentrations (25% and up), survival was indistinguishable from that of the high passage control transfectants and high passage non-transformed cells.

Each of the isolated CML-resistant cosmids contains a unique insert.

As an indicator of the heterogeneity between the inserts of the 7 cosmids, restriction fragment length polymorphism analysis and Southern hybridization experiments determined that each cosmid contained a unique insert. A representative experiment is shown (Fig. 3), in which 6 cosmids were digested with *Spe*I-*Hind*III, separated by agarose gel electrophoresis and visualized by ethidium bromide staining (Fig. 3a). *Hind*III cuts within the cosmid backbone at a single site located near one side of the insert site, and *Spe*I cuts the vector 8.4 kb from the *Hind*III site, which is about 1.8 kb from the other side of the insertion site (as diagramed in Fig. 3c). *Spe*I - *Hind*III digestion of all CML-resistant cosmids yielded differing DNA banding patterns, although all contained the expected 8.4 kb band corresponding to the *Spe*I-*Hind*III fragment of the cLHYG vector (Fig. 3). To assess whether regions of high identity were present among the inserts, Southern analysis was performed using radiolabeled DNA from an individual cosmid clone to probe the DNA from all clones. Using cosmid #2 DNA as a probe (Fig. 3b), more than 6 bands were detected among the digest products of cosmid #2, while only 2 bands (corresponding to the 8.4kb vector fragment and a variably sized vector-containing fragment) were detected in the other CML-resistant cosmid clones. The variable-sized band corresponding to the 1.8kb fragment of vector is lengthened in proportion to the location, within

the insert, of the *Spe*I or *Hind*III site nearest the vector. In addition, cosmid #6 contained a third band (2.9 kb) that hybridized to cosmid #2 probe. This third band is most likely due to rearrangements within the cosmid vector backbone, because the same band of cosmid #6 was also detected with radiolabeled probes made from the DNA of each of the other cosmids (data not shown). Additionally, when cosmid #6 was labeled as probe, no new bands were detected in the digests from cosmids #1-5 (data not shown). Equivalent experiments were performed using the same digestion and labeling procedure as for cosmid #2, but using cosmid #1 and #3-6 as probes (data not shown). These experiments indicated that each of the six CML-resistant cosmids contains a unique insert and that the insert sizes vary between 19 and 33 kb.

Sequencing of cosmid #2 indicates identity to *L. major* chromosome #36.

Cosmid #2 was selected for complete sequence determination based on initial experiments in which transfectants bearing this insert had the highest levels of CML-resistance among the isolated clones. BLAST comparison of the 31.8 kb insert sequence (Accession # AY656839) to that of the *L. major* genome indicates that the insert is homologous to a 29.9 kb region of *L. major* chromosome 36, based on the high sequence identity that exists between these sequences (BLAST scores >1,000, smallest sum probability $<1.0e^{-50}$) and on the lack of high overall identity between cosmid #2 to any other regions of the *L. major* genome. A graphical representation of the aligned regions of cosmid #2 and *L. major* demonstrates the high synteny that exists between these *L. major* and *L. chagasi* sequences (Fig. 4). The insert of cosmid #2 is predicted by analysis with GLIMMER (see methods) to code for the same 6 genes/proteins that are predicted for the 29.9 kb segment of *L. major* (Fig. 4; labeled A-F and 0790-0840, respectively). Comparison of the sequence of *L. chagasi* cosmid #2 insert and that of *L. major* chromosome 36 by GAP or BestFit programs indicates an identity of 90-94% for the predicted coding sequences when compared over their full length (Fig. 4), and 84-87% for non-coding sequences (data not shown). Queries of gene and protein databases using BLAST and PSI-BLAST searches with the predicted genes/proteins of cosmid #2 failed to identify any similarity to proteins whose activities could perturb complement lysis, i.e., proteases. Two of the

genes, B and C (Fig. 4), have predicted signal peptide sequences, indicating possible uptake to the endoplasmic reticulum, but none of the predicted proteins contain either a transmembrane spanning domain or a hydrophobic carboxy-terminus sequence that (respectively) would be typical for membrane-spanning or glycosylphosphatidylinositol-anchored outer surface proteins. Gene B has homology to an mRNA basal transcription factor subunit p52 in mice and gene D to an ADP-ribosylation factor in mice, *Candida albicans* and *Caenorhabditis elegans*; BLAST scores for identity of these genes to B and D were less than 6×10^{-5}. Blast and PSI-Blast analyses of genes A, C, E, and F failed to indicate a high level of identity to any other sequences having known or predicted functions or activities.

Discussion

These experiments demonstrate that the technique of gene complementation can be used with high passage cells that are CML-sensitive to identify genetic elements that increase *Leishmania* spp. resistance to CML. A better understanding of the phenomenological data will require a mechanistic understanding of how DNA of the cosmid-inserts acts to confer resistance to CML. Possible mechanisms include blocking the complement cascade to prevent assembly of functional membrane attack complex, shedding complement components bound to the parasite surface, and increasing the tolerance of the parasite membrane surface for assembled membrane attack complexes. Regardless of the mechanism by which resistance to complement lysis is achieved, it will also be important to determine whether gene products of the cosmids act directly to mediate the resistance, or act indirectly, i.e., by modulating levels of surface molecules that act directly to confer complement resistance). Previous studies have identified 2 surface molecules expressed in high abundance on CML-resistant cells, MSP and LPG, that appear to function in resistance to lysis by complement. MSP, a zinc protease, is believed to interrupt the complement cascade by cleaving the C3b component to an inactive form that does not support assembly of a functional membrane attack complex. LPG undergoes lengthening and changes in terminal glycosylation state, which may prevent interaction of functional membrane attack complexes with the membrane surface.

In addition to mechanistic studies, further support of a biological role for these cosmids will be supplied when individual genes that are present within the cosmid insert are characterized as contributing to CML-resistance. The sequencing of cosmid clone #2 constitutes an important step towards the identification of functional genes that are present within the cosmid insert, and will enabled the construction of a series of deletion and/or expression sub-clones to be used in transfection studies to identify those insert regions that are important for the CML-resistance conferred by the parent cosmid construct.

One potential limitation to the use of gene complementation to identify genetic elements involved in CML-resistance is the possibility that this or any episome-based methodology will lend itself to the identification of only a subset of CML-resistance genes. Specifically, the technique may not be effective in identifying certain genes whose chromosomal regulation is mirrored when expressed in episomal systems. In example, both PSA and MSPS are developmentally regulated genes whose increased abundance in metacyclic cells is due to post-transcriptional regulation of mRNA abundance. Episomal expression of PSA and MSPS appears to model “normal” expression of the chromosomally located genes in studies that have successfully used episome expression systems to identify *cis*-acting elements involved in the regulated expression of genes (Myung et al., 2002; Beetham et al., 2003). For such genes, given that they are present in the context of surrounding sequences likely to contain *cis*-acting elements involved in regulated expression, it seems unlikely that the technique of episomal complementation will result in expression of the episomal genes in growth or culture states in which the chromosomal copies are down-regulated. However, contrary to this supposition, there is always the possibility that high episome copy numbers per cell might result in transcript levels that titrate out the activity of negative regulatory factors, and thereby allow higher expression levels). In fact, in the study reported here, neither PSA nor MSPS were components of any of the identified cosmids (as was determined by Southern analysis; data not shown).

Importantly, not all developmentally regulated genes utilize a system for regulated expression that is successfully modeled in episome-based systems. For instance, MSPL, a form of MSP that is upregulated within procyclic promastigotes within cultures at logarithmic growth phase, undergoes modulated expression via post-transcriptional regulation of mRNA abundance, but this expression has not been found to be reproducible in episome-based systems (Donelson, 1995). Perhaps genes that are regulated in like fashion to that of MSPL, and not that of PSA or MSPS, will constitute the subset of CML-resistance genes that are amenable to complementation using an episome-based system.

We observed that none of the cosmid clones selected here restored CML-resistance to the level found in cells from low passage cultures at stationary growth phase. One possible explanation for this is that the product of the CML-resistance gene located within the cosmid insert may, for full activity, require other factors that are down-regulated in high passage cells; therefore, episomal re-expression of this single gene would not confer absolute CML-resistance. Such factors could include MSP, metacyclic-LPG, and PSA, molecules identified in studies as influencing parasite resistance to complement lysis (Puentes et al., 1990; Brittingham et al., 1995; Lincoln et al., 2004). Another possible explanation is that the cosmid-encoded genes having CML-resistance function may be expressed at lower levels in the serially passaged transformed cells than are the corresponding chromosome-located genes in low passage cells.

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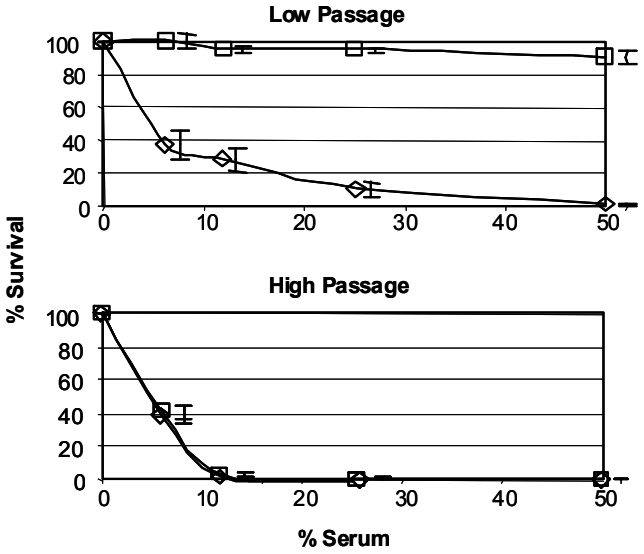

Figure 1. Promastigote resistance to complement mediated lysis.
After passage in culture for less than 5 wk (**a**) or greater than 50 wk (**b**), promastigotes from cultures at logarithmic (◊) and stationary (□) growth phase were incubated for 30 min at 37 C in human serum at 0, 6, 12.5, 25, and 50%. The percentage of cell survival was calculated as the ratio of motile cells present after incubation with, versus without, serum (see Methods). Error bars indicate the standard error of the mean of 3 experiments.

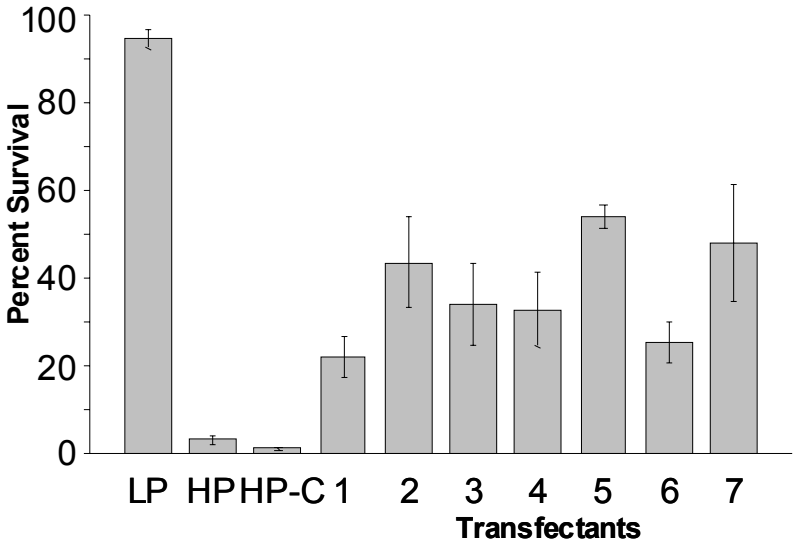

Figure 2. Cosmid-transformed promastigote resistance to complement mediated lysis. Sensitivity of promastigotes incubated in 12.5% serum to CML was assayed. Stationary phase promastigotes included non-transfected low passage (LP) and high passage (HP) cells, and high passage cells transfected with an irrelevant cosmid (HP-C) or with cosmid isolated from previous transfectants selected for CML-resistant phenotype (#1-7). The mean percent survival of the serum-selected transformants was 37% (± 11.6% at 99% confidence interval): negative controls were 2.0% ± 1.1% at 95% confidence interval; error bars indicate the standard error of the mean of 3 to 5 experiments.

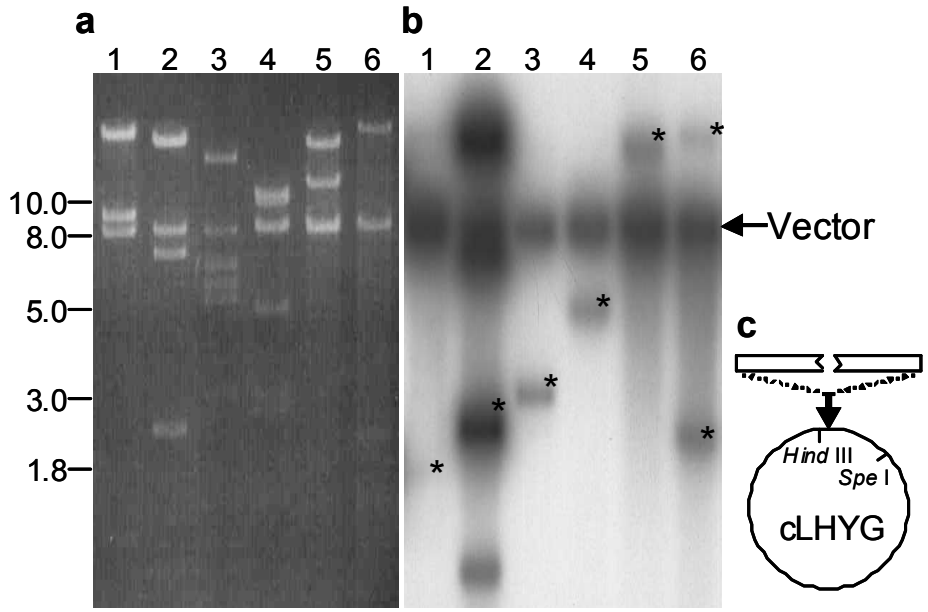

Figure 3. Diversity among cosmid inserts.
(**a**) Cosmid DNA from serum resistant high passage clones #1-6 was digested with *Spe*I and *Hind*III, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining. (**b**) The DNA within the gel was transferred to nylon membrane, probed with radiolabeled DNA of cosmid #2 (previously digested with *Spe*I and *Hind*III) and then visualized by autoradiography; bands comprised completely or partially of vector are labeled as Vector or with an asterisk (*), respectively. (**c**) A diagram of the cLHYG cosmid with an insert demonstrates the position of the vector *Spe*I and *Hind*III restriction enzyme recognition sites relative to the insert.

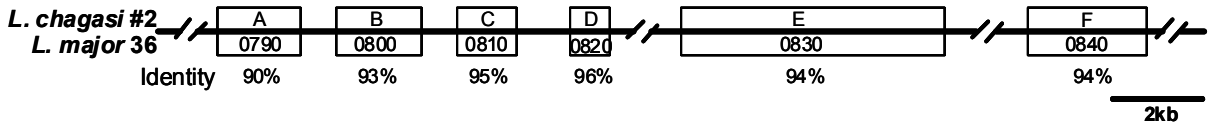

Figure 4. *L. chagasi* cosmid #2 insert and homologous region of *L. major* chromosome 36. The predicted coding sequences (boxes), non-coding sequences (line between boxes), and the percent identity shared between homologous coding sequences are as indicated. Numbers for *L. major* coding sequences, i.e., 0790, correspond to chromosome 36 coding sequence identifiers assigned by the *L. major* genome project (see Methods).

Chapter 3

Characterization of DNA sequences that confer complement resistance in *Leishmania chagasi*

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Abstract

Serial passage of axenically-cultured *Leishmania chagasi* promastigotes results in a progressive diminution in resistance to complement mediated lysis (CML), whereas high CML resistance is seen in infectious metacyclic promastigotes from the sandfly vector as well as metacyclic-like promastigotes within low passage cultures at stationary growth phase. As previously reported[1], in a screen seeking to identify novel genes involved in CML-resistance, (i) a genomic cosmid library derived from DNA of CML-resistant *L. chagasi* promastigotes was transfected into high passage (constitutively CML-sensitive) *L. chagasi* promastigotes, (ii) transformants were screened for acquisition of CML-resistance, (iii) multiple cosmid-transfectants exhibited partial CML resistance, and (iv) the sequence for one of the cosmids (Cosmid 51) was determined. The present report extends the analysis of Cosmid 51, and identifies by deletion analysis a sub-region of the cosmid insert that is critical to the CML resistance phenotype of Cosmid 51 transformants. We also report the sequence determination and initial CML-resistance activity of another cosmid that also confers partial resistance to CML.

Introduction

Leishmania spp are the etiological agents of the leishmaniases, a group of human and animal diseases ranging in severity from self-healing cutaneous lesions to potential fatal visceral infections. Transmission occurs when a parasitized blood-feeding sandfly inoculates infectious metacyclic promastigote-form parasites into a mammal or other susceptible vertebrate. Inoculated metacyclic promastigotes are phagocytosed by macrophages and other phagocytic cells of the immune system, cells in which the parasites differentiate into amastigote-form parasites that comprise the replicative stage within the mammal.

During the time between inoculation and macrophage phagocytosis, metacyclic promastigotes need to survive exposure to complement, an antimicrobial innate immune defense system present in serum. Complement involves a cascade of serum-protein binding events that can result in formation on the microbe of a membrane attack complex that kills by disrupting the outer membrane. Metacyclic promastigotes are thought to survive such complement mediated lysis (CML) through at least three mechanisms: proteolytic inactivation of the complement cascade, fast release of membrane attack complexes, and a glycocalyx structure that causes membrane attack complex assembly too distal to the parasite membrane. Three highly abundant and developmentally regulated parasite surface macromolecules are though to be involved in this resistance. Major Surface Protease (or GP63) is thought to cleave complement C3b into an iC3b-like form able to block the complement cascade while still functioning as an opsinogen able to facilitate parasite phagocytosis into macrophages via receptor CR3.[2] Lipophosphoglycan (LPG) may be involved in ineffective positioning/assembly of the MAC.[3] Promastigote surface antigen (PSA, or GP46) re-expression in promastigotes having an otherwise low PSA expression level and low CML survival resulted in greatly increased resistance to CML.[4] The high CML-resistance that is characteristic of metacyclic promastigotes does not apply to the other promastigote stages, including the procyclic, that are present in the insect host and in axenic promastigote cultures.[5-8]

The promastigote stages (e.g. the insect stages) of *Leishmania* species including *Leishmania chagasi* can be cultured *in vitro* axenically (in the absence of the fly vector). Infectious parasite cultures are established using amastigotes freshly isolated from parasitized, symptomatic animals; the amastigotes quickly differentiate into promastigotes under appropriate conditions.[9, 10] However, serial passage of promastigote cultures results in a progressive diminution in parasite capacity to infect and to resist CML.[2, 11] Based upon these observations, a gene add-back complementation study was undertaken to determine whether high passage CML-sensitive cells could be used as a genetic background against which to screen for parasites able to resist CML. The aim of the experiment was to identify novel molecules involved in CML-resistance, molecules in addition to MSP, PSA, and LPG. The initial results of the experiment as previously reported determined that when high passage parasites were transfected with cosmids containing approximately 35kb of *L. chagasi* genomic DNA, and then selected with human serum, ≈12 different cosmids were identified as conferring partial CML resistance; the sequence and annotation for one of these cosmids, Cosmid 51 (previously referred to as Cosmid 2), was also reported.[1] The present report extends the cosmid analysis.

Materials and Methods

Parasites

L. chagasi (strain MHON/BR/00/1669) maintenance in hamster and *in vitro* promastigote cultivation were as described previously.[1, 9, 10] Low passage cells were serially passaged ≤ 4 times subsequent to culture initiation with hamster-derived freshly isolated amastigotes. High passage cells were passaged for > 50 weeks; 1 week ≈ 1 passage.

Cosmids

Cosmid construction, manipulation, transfection into *L. chagasi*, and screening for CML-resistance of transfectants were as described previously.[1] A 3x coverage of Cosmid 53 sequence was determined by the Iowa State University DNA Facility utilizing a shotgun strategy by which 2kb

fragments of randomly sheared cosmid were inserted into a plasmid, and then randomly selected plasmids were sequenced; sequence gaps were filled using specific primer-directed sequencing.

Complement Assays

For complement assays, promastigotes from cultures in stationary growth phase were incubated 30 min at 37°C in PBS supplemented with pooled naive human serum, then diluted in cold PBS and viewed microscopically to assess and enumerate survivors (as described).[1]

Results and Discussion

The Cosmid 53 insert sequence of 40 kb was determined by shotgun sequencing (see methods; GenBank Accession DQ418548). BLAST comparison to *Leishmania major* and *Leishmania infantum* genomes indicated its correspondence to their chromosome 36. There is very high synteny and sequence identity between these two genomes, and correspondingly between them and that of the *L. chagasi* genomic DNA comprising the Cosmid 53 insert. We previously reported the 32 kb sequence (Accession AY656839) and chromosome 36 correspondence of the Cosmid 51 insert.[1] Although both cosmid inserts correspond to Chromosome 36, they have quite separated locations on the chromosome; Cosmid 53 corresponds approximately to *L. infantum* chromosome 36 base 284,000 to 309,000, and Cosmid 53 to base 1,190,000 to 1,230,000. Gene predictions, based upon the *L. infantum* genome annotation, indicate that Cosmid 51 comprises 1 partial (51-A) and 6 complete (51-B to G) genes, and that Cosmid 53 comprises 1 partial (53-M) and 12 complete (53-A to L) genes (Fig. 1). Table 1 lists the detail information on the gene predictions depicted in Fig. 1; in column headed "*L. infantum* V3 Chr 36", "V3" refers to genome version 3, and "Chr" to Chromosome. Many of the genes are identified as hypothetical (hp), indicating that the deduced sequence is without identifiable homologs within gene databases in the public domain.

Since parasite resistance to CML involves surface interactions with complement proteins, surface-localization is a possible characteristic of proteins that directly function in CML resistance.

None of the predicted genes in Table I encode proteins having a hydrophobic signal peptide that would signal for translation on the rough endoplasmic reticulum, a requisite for almost all surface-located proteins. However, it is also possible for CML resistance to be conferred by genes that indirectly affect the expression of surface macromolecules, or affect other aspects of cell biology including surface membrane turnover. Predicted genes 53J and K encode proteins with possible function in outer membrane events and in RNA processing; the putative mRNA processing protein is of interest since it could function in modulating expression of surface proteins whose abundance is regulated by RNA-processing events that influence RNA steady-state levels.

To further investigate sequences that function in complement resistance, a set of Cosmid 51 subclones were made by opening the cosmids using insert-specific DNA restriction endonucleases, then religating the cosmids and determining which resulting subclones had deleted the insert region targeted for removal (Fig. 1). Cosmid 51 subclones were transfected into high passage complement sensitive parasites and resulting clonal isolates were tested for CML resistance. As shown in Fig. 2, deletion of the *Bam*HI to *Bam*HI insert fragment, which deletes all predicted genes, results in abrogation of almost 70% of the CML resistance activity relative to that of Cosmid 51, while deleting the *Apa*I to *Apa*I or *Eco*RV to *Eco*RV fragments had no significant effect on relative CML resistance. The negative controls included non-transformed high passage (HP) cells and cells transformed with a cosmid from which the entire insert was removed; both controls had extremely low relative CML resistance levels (Fig. 2). Fig. 1 also depicts a similar strategy using deletion analysis for investigating Cosmid 53. However, data is not shown because these studies are still in progress.

These experiments are expanding our understanding of genetic elements and proteins that function in CML resistance. Perhaps as importantly, the studies are needed in order to test the utility of using the gene complementation strategy for identifying CML resistance genes. If the approach proves useful, it has application in the study of the CML-resistant phenotype that is a trait of almost all blood-borne microbes.

Acknowledgments

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Table 1. Cosmid gene predictions (based upon *L. infantum* genome).

Cosmid gene	*L. infantum* V3 Chr 36	Predicted gene/protein
51-A	0840	hypothetical protein (hp)
51-B	0850	hp
51-C	0860	hp
51-D	0870	hp
51-E	0880	ADP-ribosylation factor-like protein
51-F	0890	hp
51-G	0900	hp
53-A	3090	hypothetical protein (hp)
53-B	3100	succinyl-CoA ligase [GDP-forming] beta-chain, putative
53-C	3110	hp
53-D	3120	hp
53-E	3130	hp
53-F	3140	GTP-binding protein, putative
53-G	3150	ATP-dependent RNA helicase, putative
53-H	3160	glutathione peroxidase, putative
53-I	3170	exosome complex exonuclease RRP41, putative
53-J	3180	clathrin coat assembly protein-like protein
53-K	3190	pre-mRNA branch site protein p14, putative
53-L	3200	hp
53-M	3210	cullin-like protein

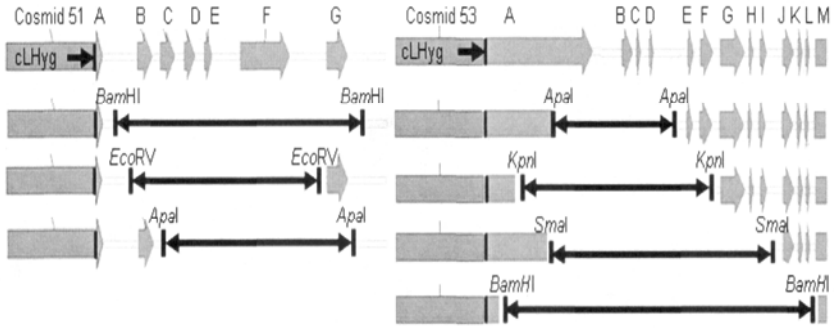

Figure. 1 Cosmid 51 and 53 gene organization and subcloning strategy.

Rectangles beneath letters indicate predicted genes, arrows indicate orientation of predicted gene transcripts, "cLHyg →" indicates vector backbone, faint narrow lines indicate regions predicted to be non-protein coding. Solid double-arrowed lines indicate insert-regions targeted for deletion in specific subclones.

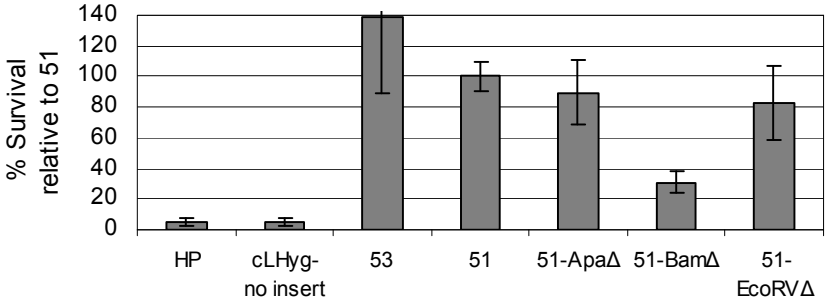

Figure. 2. Complement resistance of cells transfected with Cosmid 53, 51, and 51-subclones. The percentage of cell survival, relative to survival of cells transfected with Cosmid 51, was calculated as the ratio of motile cells present after incubation with, versus without, 12% serum.

Error bars indicate the standard error of the mean of at least three experiments.

Chapter 4

Trans-effects of leishmania vector cLHyg on Major Surface Protease (MSP) RNA and protein expression in high passage *Leishmania chagasi*

Rebecca R. Dahlin-Laborde and Jeffrey K. Beetham

Introduction

The surfaces of infectious metacyclic promastigotes, inoculated by the bite of a sand fly vector, are immediately decorated by complement proteins in the bloodstream of the mammalian host. This first line of defense by the host innate immune system is designed to eliminate invading pathogens by forming cell-lysing membrane attack complexes (MAC) on the pathogen surface and by targeting the opsonized parasites for receptor-mediated uptake by antigen presenting cells (APC) such as macrophages and dendritic cells. Metacyclic Leishmania promastigotes are capable of avoiding MAC lysis [1-4], even as they utilize serum opsonization to enhance uptake into APCs [5-7] wherein the parasites differentiate to the intracellular amastigote form responsible for infection propagation [8]. Three surface macromolecules previously described in Leishmania as possibly contributing to CML-resistance are lipophosphoglycan (LPG), major surface protease (MSP) and promastigote surface antigen (PSA). These factors are all either up-regulated at the level of RNA and protein expression (MSP, PSA) or differentially modified (LPG) as cells progress from procyclic to metacyclic promastigote stages[9-11].

In vitro, this pattern of differential expression and modification can be mimicked by growth in liquid media of low passage parasites. Parasite densities in cultures seeded at ~$1x10^6$ parasites/ml initially undergo logarithmic growth (log) comprised of procyclic and other parasite stages having low infectivity to vertebrates and low resistance to CML. After about 5 days, cultures are at stationary growth phase with densities of ~3-$5x10^7$ parasites/ml, and over the next few days cultures consist of increasing concentrations of metacyclic promastigote characterized by high infectivity to vertebrates,

high MSP and PSA expression, and differentially modified LPG. High passage cells within stationary phase cultures fail to exhibit these characteristics; they have low infectivity, low PSA and MSP levels, and non-differentially modified LPG.

Based on the surface localization of these previously described virulence factors and the positive correlation of their expression with CML-resistance (reviewed in chapter 1), and their loss in the high passage cells used as the background for our complementation rescue of CML-resistance experiments, a reasonable hypothesis is that the restored CML-resistant phenotype displayed by HP cosmid clones may involve restored expression of one or more of these putative lytic-resistance factors. Because the state of knowledge and reagents available for analyzing MSP were more advanced than for PSA, efforts to test the hypothesis focused on characterizing MSP levels. As reviewed in Chapter 1, the proposed mechanism by which surface MSP mediates CML-resistance is via its proteolytic cleavage of complement factor C3 into the CML-inactive form iC3b, a form that retains opsogenic properties. MSP occurs in 3 isoforms in low passage (LP) promastigotes; MSPL (log) is highly expressed in logarithmic (log) phase cultures and remains detectable on the cell surface in stationary phase cultures, MSPC (constitutive) is expressed at low but constant levels in log and stationary phase cultures and MSPS (stationary) is expressed in mid-log to stationary phase cultures. A combination of expression of these isoforms results in a high abundance of MSP in stationary phase cultures. For this reason, and the abundance of MSP expression on metacyclics, the CML-resistant cosmid clones were screened for re-expression of MSP RNA and protein.

Materials and Methods

Parasites

Infectious *L. chagasi* amastigotes (strain MHOM/BR/00/1669, originally isolated in Brazil from a patient with visceral leishmaniasis) were maintained in Golden Syrian hamsters by serial passage as described [12]. Amastigotes were differentiated into promastigotes, and the promastigotes were subsequently cultured, using supplemented Modified minimum essential media (HOMEM) as

previously described [10]. Promastigote cultures seeded at 1.0 x 10^6 cells/ml were split to 1.0 x 10^6 cells/ml seven days later, a time that corresponded to 2-3 days after reaching stationary growth phase with a density of 2-4 x 10^7 cells/ml. Logarithmic and stationary phases of cultures were determined by cell morphology and culture density as described [13]. Cells cultures were considered low passage (LP) if serially passaged for ≤5 wk. High passage cells (HP) used in the following experiments were passaged for >50 wk.

Transfection and clonal isolation

Transfection of high passage promastigotes by electroporation of cosmid DNA was largely as described [14], excepting that electroporation was in 75% electroporation buffer (21 mM HEPES pH 7.5, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose) and 25% CytoMix (120 mM KCl 0.15 mM $CaCl_2$, 10 mM K_2HPo_4, 25 mM HEPES, 2 mM EDTA, 5 mM $MgCl_2$). Electroporation in 0.4 cm cuvettes used the following electrical constraints: 2 kV and 25 µfd. High passage promastigote cultures in late logarithmic growth phase (0.8-1.0 x 10^7 cells/ml) were transfected using 8x10^7 cells per transfection reaction. Following transfection, cells were incubated 72 hr in 1X M199 media (Cellgro, Herndon, Virginia) to allow recovery. Cells were pelleted by centrifugation for 5 min at 1,000 g, resuspended in 500 µl HOMEM and spread onto semi-solid media containing M199 medium (Invitrogen) supplemented with, or without, 50 µg/ml Hygromycin B (Sigma, St. Louis, Missouri). Colonies were isolated from plates 1-3 wk later, and individual isolates were cultured in HOMEM supplemented with 10% heat inactivated-fetal calf serum and 0 or 50 µg/ml Hygromycin B. High passage control cultures, “mock transfectants” were generated by identical transfection procedures as described above, except that PBS alone was used in the transfection reaction in place of cosmid DNA and the resulting clones were isolated on plates and expanded in HOMEM supplemented with 10% heat inactivated-fetal calf serum in the absence of hygromycin selection.

Isolation of high passage cosmid transfectants in the absence of drug selection

High passage promastigote transfectants, clonally isolated on plates and expanded in liquid culture in the absence of Hygromycin B selection, were split into parallel cultures at a concentration of 1.0 x 10^6 cells/ml including, 0 µg/ml hygromycin or 50 µg/ml hygromycin. Cell density was determined, by enumeration on a hemocytometer, for the parallel cultures on each of 7 days of growth. Non-drug selected, HP cosmid clones (-) were chosen for further study from those cultures that exhibited comparable growth characteristics in matched cultures selected in drug, indicating the presence of a cosmid conferring drug resistance; growth characteristics included reaching stationary growth phase by day 6 in culture and a maximum density greater than 3.0 x 10^7 cells/ml.

RNA

Total RNA, isolated from *Leishmania chagasi* promastigote cultures using TRI reagent (MRC, Cincinnati, OH) according to manufactures instructions, was separated by agarose-gel electrophoresis and transferred to nylon membrane as described [11]. Following hybridization of DNA probes, blots were washed at high stringency. DNA probes were labeled by random-primer incorporation of radionucleotide using Ladderman Labeling kit Takara, Japan). An MSPS specific DNA probe was generated by polymerase chain reaction (PCR) using the primer pair 5' cctcgcttatacacccac 3' + 5' tatagagagccacgagg 3'; template was plasmid containing sequence encoding MSPS (GenBank Accession number M80669). A tubulin specific probe was PCR amplified from plasmid template containing sequence for α-tubulin using the primers 5' ggcgaattcatgcgtgaggctatctgcat 3' + 5' ggcaagcttttagtactcctcgacgtcc 3'. Radioactivity on blots was visualized by autoradiography.

qRT-PCR

Total RNA was quantified for concentration and purity by spectrophotometry (NanoDrop, Wilmington, DE). Samples were DNase treated and a test plate was run to determine the optimal dilution range (lacked inhibition and exhibited LOG-linear behavior and amplification efficiencies

>80%). All DNase treated RNA samples were diluted in nuclease free water to the ideal concentration and placed in duplicate into 96 well plates. The negative, no template control wells contained nuclease-free water in place of RNA sample. A one-step qRT reaction was run for 40 cycles using the SuperScript III Platinum SYBR Green kit (Invitrogen, Carlsbad, CA) and the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) and relative quantification of mRNA targets was analyzed using the GeneAmp 5700 software. Additionally, α-tubulin mRNA levels were used as an internal control. To evaluate MSPS mRNA levels in total RNA samples, primers specific to the MSPS 3' UTR were designed, resulting in a 125 bp amplicon, contained within the region of the MSPS 3'UTR that was PCR amplified for use as a Northern probe (see RNA).

Protein analysis

Parasites were pelleted by centrifugation (2000g, 10min), washed twice in PBS, resuspended in 0.1%-TX100-supplemented sodium phosphate buffer pH 7.2, and lysed by 3 freeze-thaw cycles. Equivalent amounts of protein were added per lane as determined via BCA assay. All lysates were separated via SDS-PAGE and electro-semi-dry-transferred to PVDF membranes using standard buffers. Blots were probed with a 1:2000 dilution of primary polyclonal sheep antisera to MSP that detects all forms (MSPL, MSPC and MSPS) and was raised against purified *L. chagasi* MSP as previously described [15]. A horseradish peroxidase-conjugated anti-goat secondary antibody (Pierce) was used at a 1:20,000 dilution. Antibody binding was visualized via chemiluminescence.

Results

MSPS RNA and protein in CML-resistant cosmid transfectants

Analysis of mRNA in the CML-resistant cosmid transfectants yielded the surprising finding that MSPS mRNA steady state levels were substantially higher in the transfectants than in the control HP cells (Fig. 1A). As expected based on prior studies by ourselves and others [11, 16] MSPS levels decreased ~ 30 fold in control HP cells, relative to LP stationary cells (Fig. 1A). These results indicate that MSPS expression was at least partially restored in the CML-resistant cosmid transfectants. It was

previously determined that none of the cosmid inserts corresponding to these cosmid transfectants contain MSPS or PSA coding sequence. Therefore, the restored MSPS expression evidenced here suggests that the cosmids within these transfectants are somehow acting in *trans* to increase steady state levels of MSPS mRNA derived from chromosomal genes.

The same cultures/cells analyzed for MSPS RNA were also analyzed for MSP protein levels by Western blot analysis using an MSP polyclonal anti-serum that detects all three protein isoforms (MSP-L, C and S) (Fig. 1C). As shown, these levels paralleled the pattern of mRNA expression seen in Fig. 1A. Similar to RNA expression patterns, MSPS protein expression is detectable in stationary LP control cultures, but is expressed at very low levels in stationary HP control cultures (Fig. 1C). LP non-transected promastigotes have been shown to display 2 MSP bands on western analysis, a predominant 63 kDa and less predominant 59 kDa band which increase in expression as cultures progress from log to stationary growth phase (Fig. 1B). Again, all of the stationary phase HP cosmid clones showed increased levels of MSP expression. However, nearly all cosmid clones express only the 59 kDa product, differing from LP control cells, while one cosmid clone, 52, displays only the 63 kDa product. These results indicate that the HP cosmid clones are re-expressing both MSPS RNA and a form of MSP protein in stationary phase of growth. However, the detectable expression of only one product for each cosmid transfectant suggests the possibility that the resulting MSP protein may not be correctly processed, glycosylated or transported for anchoring on the membrane surface (and therefore possibly remaining within an intracellular location) or suggesting that the form of MSP in almost all of the transfectants is correctly processed and is equivalent to the 59kDa MSP that predominates in LP cells before cultures enter stationary phase Related to these questions, it is not know if the MSPS protein product is processed to an active zinc protease, able to cleave C3 to iC3b, as is seen on the surface of LP promastigotes.

One additional finding of great interest derived from experiments that included a negative-control cosmid transfected with the cLHYG vector without insert (Fig. 1C, Vector). Clonal isolates of

HP cells, transfected with the empty cLHYG vector, have routinely been included as a negative control in CML assays due to their constitutive CML-sensitivity in log and stationary phases of growth (data not shown). Because of this CML phenotype, it was surprising to discover that the vector HP clones expressed MSP protein levels similar to the CML-resistant HP cosmid clones. One possible hypothesis is that the transfection procedure, or subsequent clonal isolation, may result in selection of HP cells displaying restored MSP phenotype. However, HP mock-transfected control cells used in these experiments were exposed to the same transfection procedure (PBS, no DNA) and clonal isolation on semi-solid media as the cosmid transfected HP clones, yet displayed no evidence of MSPS-RNA or -protein re-expression. To confirm the observation of MSPS RNA expression in HP cosmid transfectants, a second set of clonal isolates were examined by Northern analysis (Fig. 2). Again, MSPS RNA is highly expressed in LP control as compared to HP control samples, and all cosmid clones express some level of MSPS RNA, including the cLHYG vector control, albeit at lower levels than the previous Northern experiment.

MSP RNA in CML-sensitive cosmid transfectants

The observation that HP, cLHYG vector control transfectants display restored expression of MSPS RNA and protein suggests that all HP cosmid transfectants will express MSPS RNA, regardless of the sequence contained within individual cosmids. To test this hypothesis, MSPS levels were analyzed in HP cells transfected with cosmid constructs (IR1-11) previously determined not to confer complement resistance. In stationary phase cultures at day 6 of growth, MSPS RNA expression relative to HP control samples was high in 10 of the 11 IR clones and in the LP control. Only clone IR1 failed to show MSPS RNA expression at levels higher than the HP control. However, when IR1 cultures were assayed over days 5, 6 and 7 of growth, MSPS RNA exhibited a dramatic increase at day 7 (Fig. 3B). Clones IR5 and 7 were also subjected to the multi-day RNA analysis; similar to IR1, MSPS RNA levels within these clones also increased as cultures spent more time in stationary phase.

MSP RNA in non-transfected, drug selected, high passage promastigotes

Collectively, these results show that MSPS RNA is stabilized in all HP cells transfected with a cLHYG cosmid, regardless of the presence or absence of specific insert sequences. Additional experiments were undertaken that sought to determine whether MSPS RNA upregulation was due to the cosmid, or to the selection process and drug exposure. HP, non-transfected, clonal isolates were grown for multiple weeks in the presence of increasing concentrations of Hygromycin B and were assayed by RT-PCR, using primer sets specific to MSPS 3'UTR sequence, for expression of MSPS RNA. HP, non-transfected cells were grown in culture media containing either 0, 5 or 10 µg/µl of Hygromycin B. Drug selected HP cultures displayed hindered growth patterns as compared to non-selected HP controls, with culture densities reaching a lower peak concentration (Fig. 4A, representative growth curve). Cultures were selected between 3-6 weeks with drug, and assayed weekly for MSP RNA expression. RT-PCR analysis shows low to non-detectable levels of MSPS RNA expression in the HP, drug selected cultures as compared to HP control cells, the highest increase being 1.14 fold over HP control, while a LP control culture showed a 47.6 fold increase over HP control. These data suggest that non-transfected, HP cells do not display the phenotype of stabilized MSPS when exposed to the selective pressure of Hygromycin B in culture.

MSP RNA in cosmid transfected, high passage promastigotes, with and without drug selection

To examine the effects of two variables, presence of cosmid and drug selection, on the stability of MSPS RNA in HP promastigotes, HP cells derived from a single culture were transfected with either cLHYG 51, cLHYG 51Δ, cLHYG vector (no insert) or mock transfected (HP control). The resulting transfectants were clonally isolated on plates in the presence (+) or absence (-) of Hygromycin B at 50 µg/ml. Cultures derived colonies on the no-drug plates were verified for presence of cosmid by testing for Hygromycin resistance (see Methods). In total, 6 treatment groups were analyzed: 51 (+ or – drug), 51Δ (+ or – drug), cLHYG (+ or – drug), and 2 non-transfected controls HP and LP (- drug). These treatment and control groups were expanded in liquid culture for 7 days, and

MSPS RNA levels were determined by RT-PCR for those days corresponding to late log through stationary culture phase.

The results of one experiment are displayed as the fold increase of MSPS RNA expression over the HP control group (Fig. 5). All HP, cosmid transfected clones exhibit a trend of increased MSPS RNA expression (2-3.5 fold over HP control) on day 7 of culture growth. A similar pattern of expression is displayed by the LP control samples, increasing nearly 60 fold over HP control, and expressing at much higher levels than the cosmid transfectants, with the largest increase in expression occur one day earlier in culture growth. Additional comparison of similar cosmid insert transfectants, differing in their exposure to drug, 51 (+ or – drug), 51Δ (+ or – drug), cLHYG (+ or – drug), examined the possibility of drug selection effecting the stabilization of MSPS RNA in transfectants. The data on days 5-7 show similar patterns of MSPS RNA expression in all of the treatment groups, regardless of the presence or absence of drug selection. These data collectively suggest that the phenotype of MSPS RNA stabilization is detectable in HP cosmid transfectants, regardless of the presence or identity of insert within the cosmid, and regardless of presence or absence of drug selection.

Discussion

Serious consideration must be given regarding the origin of the cosmid vector in attempting to address the observed phenomenon of MSPS stability in cosmid transformants. The pedigree of the cLHYG cosmid vector begins with the finding that selection of *Leishmania major* promastigotes with methotrexate, a potent inhibitor of dihydrofolate reductase, results in a resistant cell line. This line overproduces the bifunctional protein dihydrofolate reductase-thymidylate synthase (DHFR-TS) and amplifies a 30 kb region of DNA containing the coding region for *DHFR-TS* at a copy number ~100 times that of wild type promastigotes in culture [17]. Initial studies identified the presence of both stable and unstable amplified DNA in these resistant cell lines and found that relieving drug pressure did not cause the copy number of *DHFR*-TS to drop to pre-selection levels. They concluded that only

the unstable DNA existed as extrachromosomal units, lost during cell division, and the stable DNA was retained by insertion into a chromosomal locus as a repetitive array [17]. Later work determined that both the stable and unstable DNA elements exist as extrachromosomal, circular DNA, generated from the chromosomal locus of *DHFR-TS,* and that the chromosomal coding region remains unchanged [18]. Construction of the cLHYG cosmid vector utilized the up and downstream regulatory elements of the *DHFR-TS* coding region for their ability to confer episomal, stable replication in *Leishmania* promastigotes. The cLHYG construct retains the 5' and 3' *DHFR-TS* UTR sequences, while the protein coding region is replaced by coding region for the hygromycin B resistance marker [19].

Gene duplication and gene amplification are extremely common events for nearly all organisms studied. There are countless references citing gene amplification as a mechanism of response to conditions where increased gene expression is necessary to circumvent a negative effect on the organism (reviewed in [20]). A less immediate consequence of gene amplification is that it can facilitate evolution of function to occur through mutation, since a required gene/protein activity can be maintained through the presence of the original gene, thereby enabling mutations that may potentially negatively affect gene/protein function to accumulate in the duplicated gene without detriment. Amplification has been described as a device to extend the range of gene expression to handle extreme conditions [21], but it has an advantage over structural DNA changes such as point mutations, it is reversible and effects of amplification can diminish in the absence of continued stress or selection. Gene amplification has been linked to heat stress tolerance in bacteria, insecticide resistance in mosquitos, drug resistance in yeast and herbicide resistance in plants, a few of many examples (reviewed in [22]). For these reasons, the discovery of amplification of the *DHFR-TS* gene in *Leishmania* promastigotes selected in methotrexate was not without precedent [17]. The unusual feature of this system is that the amplified genes are contained on circular episomal DNA which remains stable in the absence of drug selection [18]. These discovery that the amplified genes were contained on circular episomal DNA which remain stable in the absence of drug selection [18],

allowed construction of stable shuttle vectors that have significantly added to the resources available for the study of *leishmania* parasites.

Work in our lab has employed the resulting shuttle vector system to generate a complementation library that could be stably transfected to promastigotes, in an attempt to restore specific phenotype [23]. Through related experiments, we have identified a phenotype of restored stabilization of MSPS RNA levels in cells normally expressing low MSPS. These results intriguingly suggest that Leishmania episomes may function in *trans* to affect expression levels of genes not encoded on the episome. The findings contained within this work may represent the first example of an episome-derived shuttle vector that acts in *trans* on RNA levels; we have found no other reports of this phenomenon in the literature. Additional studies are underway that seek a more complete understanding of this phenomena in terms of the mechanism as well as of the sequence required; *DHFR-TS* flanking regions that derive from the originally described episome and are retained within the cosmid would seem to be likely candidates for involvement in the process. Information gained from such studies may provide essential knowledge toward gaining a better understanding of regulation in these organisms.

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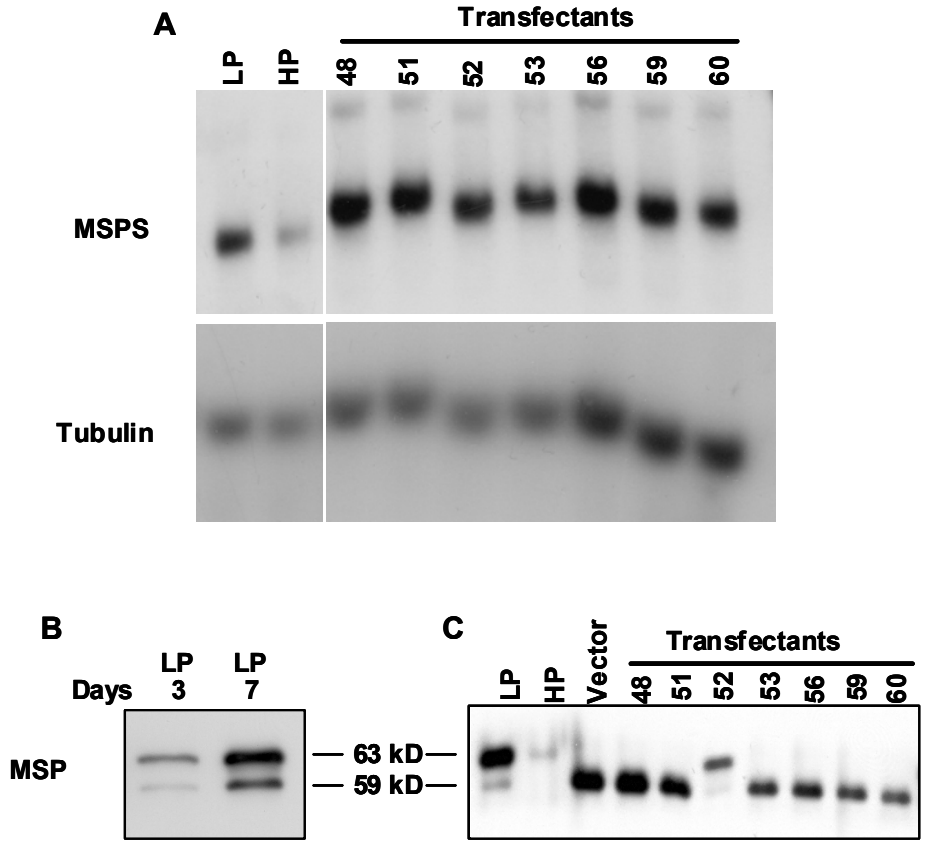


Figure 1. MSPS RNA and protein in *Leishmania* CML-resistant cosmid tranfectants
Total RNA and total protein were isolated from log ans stationary phase cultures of non-transfected low passage control (LP), mock transfected stationary phase cultures of high passage (HP) control, and from stationary CML-resistant transfectants #48-60. (A) RNA was subjected to Northern analysis using MSPS-specific (upper panel) or α-tubulin-specific (lower panels) DNA probes, then visualized by autoradiography. (B) Protein from LP log (day 3) and stationary (day 7) phase cultures and (C) stationary phase LP control, HP control and CML-resistant transfectants #48-60 were subjected to Western analysis of MSPS levels using MSP polyclonal anti-serum that detects all three protein isoforms (MSP-S,C,L).

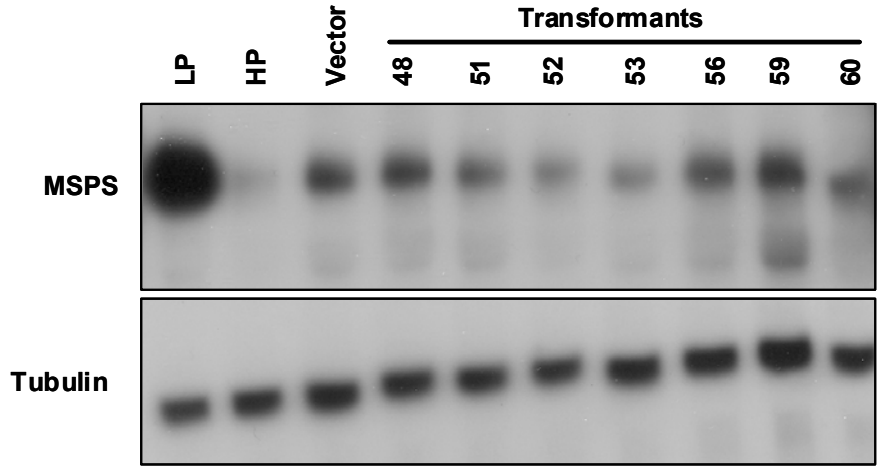


Figure 2. MSPS RNA in *Leishmania* CML-resistant cosmid transfectants
Total RNA was isolated from stationary phase cultures of non-transfected low passage control (LP), mock-transfected high passage control (HP), cLHYG vector transfected high passage (Vector) and cosmid transfected HP (#48-60) promastigotes. RNA was subjected to Northern analysis using MSPS-specific (upper panel) or α-tubulin-specific (lower panel) DNA probes, and visualized by autoradiography.

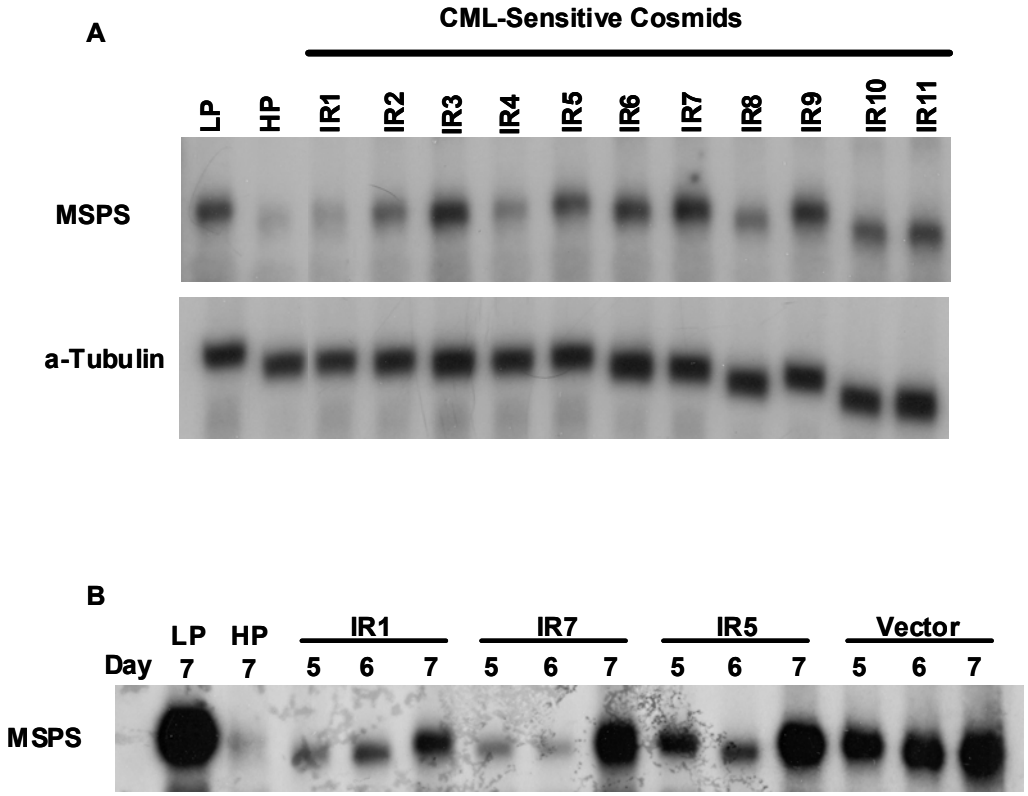
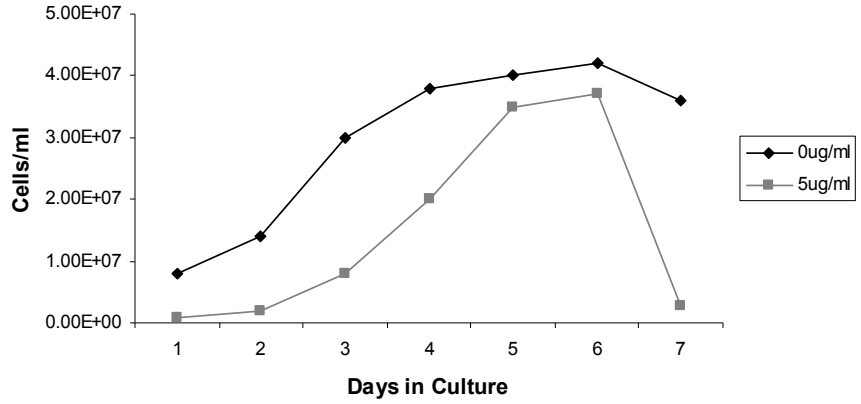


Figure 3. MSPS RNA in *Leishmania* CML-sensitive cosmid transfectants
(A) Total RNA was isolated from stationary phase cultures of non-transfected low passage control (LP), mock-transfected high passage (HP), and stationary phase CML-sensitive, cosmid transfected (IR) promastigotes. (B) Total RNA was isolated from late log and stationary phase cultures (days 5-7) of non-transfected low passage control (LP), mock-transfected high passage (HP), stationary phase CML-sensitive, cosmid transfected (IR), and cLHYG vector transfected (Vector) promastigotes. All RNA was subjected to Northern analysis using MSPS-specific or α-tubulin-specific DNA probes, then visualized by autoradiography.

A



B

Weeks in Culture	Hygromycin B µg/ml	Fold increase over HP
1	5	1.06
2	5	0.94
3	5	0.98
1	10	0.70
2	10	0.56
3	10	0.93
4	10	1.14
5	10	0.78
6	10	0.58
LP	0	47.67

Figure 4. Drug selection of high passage *Leishmania* promastigotes and MSPS RNA expression. High passage (HP) cultures of non-transfected promastigotes were selected in 0, 5 or 10 µg/ml Hygromycin B for 3-6 weeks. (A) Growth curves were determined over 7 days of growth in culture, (shown, a representative growth curve of drug selected HP promastigotes). (B) Total RNA was isolated from drug selected HP and non-selected HP and low passage (LP) cultures for analysis by RT-PCR with MSPS 3'UTR specific primers. Values presented represent the fold increase over HP, non-selected promastigote cultures.

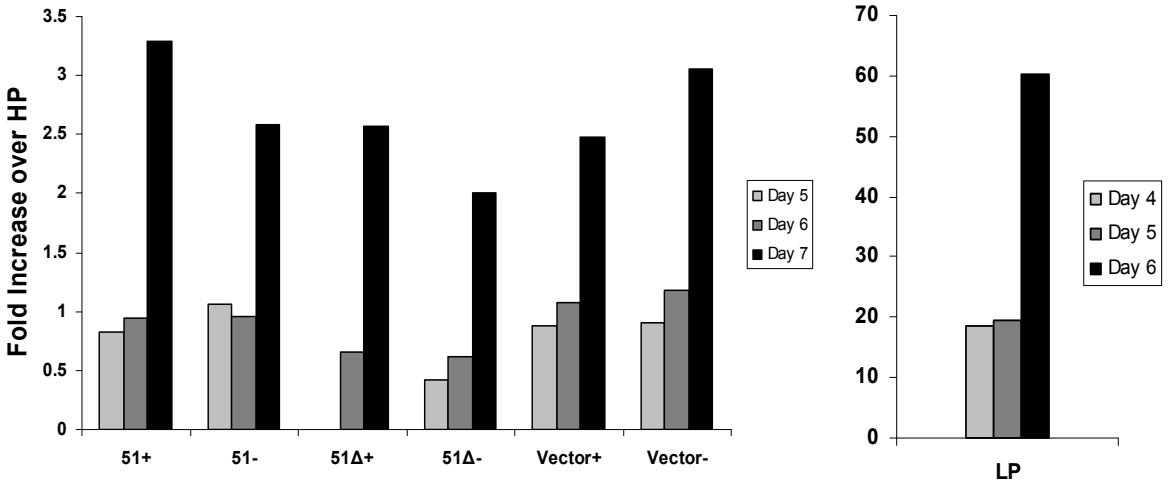


Figure 5. MSPS RNA in *Leishmania* cosmid transfected promastigotes.
Total RNA was isolated from late log and stationary phase cultures (days 4-7) of mock-transfected high passage (HP), non-transfected low passage (LP) and three high passage cosmid transfected promastigote cell lines (51, 51Δ and cLHYG Vector). All cosmid transfected lines were clonally selected on plates and expanded in the presence (+) or absence (-) of 50 µg/ml Hygromycin B in liquid culture. Expression of MSPS RNA was analyzed by RT-PCR using MSPS 3'UTR specific primers. Results are reported as the fold over HP non-transfected promastigote MSPS expression.

Chapter 5

General Conclusions

The work presented in this dissertation describes efforts to characterize the process of metacyclogenesis, the generation of infectious parasites within the sand fly vector. Many factors must converge to allow for proper differentiation of the promastigotes to the various parasite forms necessary to establish an infectious population within the vector, available for transmission to a host upon blood feeding. The main focus of this research was to obtain a deeper understanding of the factors involved in a parasite's ability to resist lysis by complement factors in host serum (CML). Initial experiments utilized a genomic complementation system to identify elements capable of conferring a phenotype of CML-resistance to high passage promastigote cell lines, which normally exhibit a CML-sensitive phenotype. These screening experiments yielded a number of cosmid clones which conferred increased CML-resistant phenotype to these high passage cell lines. Further examination has identified a more defined region of DNA sequence contained within the cosmid, capable of conferring phenotype. Studies continue within our group to further characterize cosmid inserts and to attempt to define mechanisms by which these insert regions confer phenotype.

An interesting observation during these initial studies led to a more defined examination of the cosmid vector used in the construction of the genomic library. It was determined that high passage cells transfected with the cLHYG cosmid vector express increased levels of MSPS RNA and protein. MSP is a virulence factor normally unregulated in infectious promastigotes, a pattern of regulation that is lost during serial passage in culture media. This restoration of expression may represent the first example of function assigned to the episomal cosmid that is not related to insert sequence contained within the vector. Additional studies aim to define a region within the cosmid vector that is capable of conferring the phenotype of unregulated MSPS RNA. Studies also aim to characterize the ability of the cosmid to confer up-regulation to other species of RNA and protein, to determine if transfection with the cosmid has a more global effect than was originally observed.

Determining a mechanism for the increased expression in cosmid transfected cells may result in a significant contribution to the overall understanding of the regulation occurring in *leishmania* promastigotes. These types of advances will be critical in the pursuit of developing new treatment and vaccine options to fight the global crisis of *Leishmania* infection.

Appendix 1

Further characterization of cosmid 51 deletion constructs

The initial experiments involving the *L. chagasi* genomic complementation library transfected into HP cell lines are detailed in chapters 2 and 3. The following is a description of the continued efforts to characterize the elements conferring CML-resistant phenotype contained within the cosmid 51 insert. As discussed in chapter 3, a deletion series of subclones was constructed for cosmid clones 51 and 53 by opening the cosmids with endonucleases, which cut multiple times within the insert but not the vector, and re-ligating the terminal 5' and 3' sites, eliminating various amounts of insert sequence. Complement assay data presented for clone 51 (Apa Δ, Bam Δ and EcoRV Δ) showed that the Bam Δ resulted in a 70% reduction in CML-resistance versus full insert 51, while the Apa Δ and EcoRV Δ constructs had CML-resistance equal to that of cosmid clone 51. Based on the outcome of CML assays for the cosmid 51 deletion series (chapter 3, figure 2), 51 EcoRV Δ was chosen for further analysis. This cosmid was chosen due to its retention of CML-resistant phenotype despite elimination of 23 kb of insert sequence, and because it contains 78% of the predicted open reading frame (ORF) for gene F. The complete Gene F ORF is 2.3kb long and contains 2 regions having homology to zinc finger domains in a related species, *Trypanosoma cruzi*.

A series of constructs were designed to isolate a smaller region of insert capable of conferring phenotype. The rational for testing DNA regions in the pXG vectors in addition to the cLHyg cosmid vector was the smaller size of the pXG vectors, availability of additional restriction enzyme sites for cloning purposes and that pXG vectors provide the appropriate processing elements for *trans*-splicing and polyadenylation of *leishmania* polycistronic transcripts. Additionally, the presence of the 18S promoter on one of the pXG plasmids would have the potential to drive higher transcript levels and thereby (possibly) increase the CML-resistant phenotype. Since higher expression rates sometimes yield negative (worse) results, the promotor-less version (pXG) was also tested.

Existing vectors were modified for use in this experiment, resulting in three recipient constructs (Figure 1). An existing vector, pXG-18S-Neo, was chosen for use in constructing vectors pXG-18S and pXG-Hyg. The pXG-18S-Neo vector is commonly used as an expression vector in *leishmania*, and contains an 18S promoter to drive higher levels of transcription and (flanking of the insert site) the appropriate elements for splice acceptor leader and poly-A addition to the 5' and 3'ends of transcripts derived from the insert. Because anecdotal evidence from our and other labs has shown that high passage promastigotes resist neomycin when selected on semi-solid media plates (but, curiously, are neomycin sensitive in liquid culture) the coding region for neomycin phosphotransferase resistance was replaced by the coding region conferring Hygromycin B resistance to form plasmid pXG-18S-Hyg; HP promastigotes are highly sensitive to hygromycin both when grown on semi-solid medium during clonal selection and when grown in liquid culture. Plasmid pXG-Hyg was constructed by removing the coding region for the 18S promoter from pXG-18S-Hyg using restriction sites that flanked the 18S promotor. Finally, the cLHYG cosmid vector component of the cosmid-constructs used in the initial screening experiments was modified by repairing the *BamHI* cloning site, which was disabled during the packaging of cosmid library DNA, by a *Sau*3A to *Bam*HI ligation of insert to vector.

Various regions of cLHYG 51 EcoRV Δ insert were isolated by PCR amplification and cloned into some or all three of the recipient vectors (depicted in Fig.1) to determine which portion of the 51 EcoRV Δ insert is responsible for the observed CML-resistance in HP cultures, and to validate use of the pXG vectors. Regions tested were the (i) 2.1kb EcoRV 5', (ii) 6.2kb EcoRV 3' and (iii) the 8.3kb EcoRV 5'+3' (Fig. 1A-C). The CML-resistance function of the EcoRV 5'+3' fragment was assessed in transfectants bearing all three vectors-constructs, while the EcoRV 5' and the EcoRV 3' fragments were assessed only in transfectants bearing pXG-18S/Hyg-constructs.

HP cultures transfected with the pxG-18S-Hyg vector that contained either the EcoRVΔ 5' or the EcoRVΔ 53' inserts failed to exhibit increased CML-resistance (Fig. 2). Similarly, HP cultures transfected with the pxG-18S-Hyg or the pXG/Hyg vectors that contained the 8.3kb EcoRVΔ 5'+3'

insert failed to exhibit increased CML-resistance. As we had seen in earlier screens (see Chapter 3), only cells transfected with the cLHyg vector that contained the 8.3kb EcoRVΔ 5'+3' insert exhibited increased CML-resistance.

These results suggest that the pXG-based vectors may be inappropriate for use as expression vectors in these experiments. The reasons for these aberrant results using the identical inserts in different vectors are unknown, but several possibilities exist. The initial rational for employing the pXG-18S-Hyg vector for these experiments was to use the 18S promoter to drive for higher transcript levels of the insert DNA, potentially leading to higher levels of RNA translation and greater observable CML-resistance phenotype. One possibility is that the pXG vectors do result in higher RNA transcript levels as compared to the cosmid, either by increasing the transcription rate from the plasmid or increasing the plasmid copy number, but that higher transcript copy number may act to decrease the stability of the resulting RNA, ultimately resulting in lower CML-resistance phenotype. These questions will be addressed through a series of Southern experiments to determine vector copy numbers and Northern experiments to determine transcript abundance for a specific gene product contained within the insert sequence. Another possible reason for the lack phenotype with the pXG-vectors could relate to a change in intracellular localization of the plasmid vector compared to the cosmid vector, resulting in a change in RNA transcription rates or RNA stability. Studies are currently underway to isolate episomal units and determine their localization within the promastigote.

As previously discussed, sequence comparison of cosmid clone 51 to *L. major* database sequence indicated the presence of a 2.3kb coding region located within the last 7kb of the insert. This predicted ORF (51 F) contains regions with homology to zinc finger domains, indicating a possible DNA binding function. This pORF is partially contained within the 3' fragment of the 51 EcoRVΔ construct, with the initial 519bp of the coding region truncated by the *Eco*RV restriction site used in generating the Δ construct. Figure 3 (A) illustrates the region of gene F that is eliminated by the ligation of the 5' and 3' *Eco*RV sites, eliminating the intervening sequence. To test the hypothesis that the partial coding region F retained in the EcoRVΔ constructs is involved in the restored CML-

resistance in HP transfected cells, inserts containing the full F coding region were generated by PCR and ligated into the pXG-18S-Hyg vector. Figure 4 shows that pXG-18S-Hyg F+ constructs confer intermediate levels of CML-resistance in comparison to cLHYG vectored full 51 insert (51) or the cLHYG vectored 51 EcoRV Δ. This increased resistance in comparison to negative controls suggests a possible involvement of the products of gene F in restoring CML-resistance.

Extreme caution must be taken in interpreting this positive results regarding gene 51F, given the results described in Fig. 2 wherein an insert that yielded function in vector cLHyg did not yield function in the pXG vectors. Therefore, ongoing experiments seek to validate the role of gene 511F via expression-constructs that utilize cLHyg. The coding and 5'-flanking regions of F within EcoRV 3' are being restored, which will result in the restoration of the full coding region of gene F and will provide the upstream splice acceptor site necessary for correct processing (Fig 3B). This construct will be ligated into the cloning site of the three recipient vectors described in Figure 1 (pXG-18S-Hyg, pXG-Hyg and cLHYG), and transfected in CML-sensitive HP cells, and resulting transfectants ill be assessed for CML-resistance.

Characterization of complement assay, time course experiments

Following transfection with the genomic cosmid library, HP cells lines were exposed to pooled human serum to select for CML-R transfectants (see methods, chapter 2). The surviving cells were plated to yield clonal populations, which were isolated and expanded as clonal isolates in liquid culture. There were ~100 clonal isolates obtained from the original screen which were stored in liquid nitrogen as log phase promastigotes. From these 100 clonal isolates, 46 were initially examined by complement assay (see methods, chapter 2) to determine their level of CML-resistance or sensitivity. From these original 46 clones screened by CML assay, a total of 29 clones were chosen for further analysis in a series of 2 to 5 assays (Figures 1A and 1B). During the course of these initial assays, observations were made regarding the large variability of resistance levels exhibited by the clonal isolates between experiments, as indicated by the large error bars reported in Figure A and 1B.

The most significant observation in these experiments was that the growth rates of cultures, measured by calculating cell density on consecutive days, differed significantly between LP and HP cultures and transformants. To address this, complement assays were performed on days 3-7 of growth in culture for all samples, running all assays as time course experiments. The resulting data demonstrated that the various cultures reached stationary phase on different days and that this correlates with peak levels of complement resistance in LP and transformant cultures (Figure 2). The growth curves for LP and HP cultures follow a similar trend, reaching a similar peak density between days 6 and 7 of growth. HP cells transformed with cosmid 51 also follow a similar growth curve during early and late log phase (days 3-4), but reach their maximum culture density earlier (day 5) and grow to a lower maximum density than non-transformed cells. Both LP and 51 transformed HP cells display maximum levels of complement resistance at time points corresponding to maximum culture density. One additional observation is the apparently high levels of complement resistance observed in HP and 51 transformant cultures in the log phase of growth (day 3). Retrospective data surveys have shown that these values are artificially inflated due to higher levels of cell death in the 0% serum controls which are incubated at 37 C for 30 min for use as a comparative control to the treatment groups. The results of these experiments emphasized the importance of performing the complement assays when cells are at the maximum cell density to detect maximum levels of complement resistance. To assure that each data set represents the peak of CML-resistance, all assays are now performed on time courses and the peak data are reported. These considerations may be applied to the original assays of transfectants (Figures 1A and 1B) to explain the large variability observed between experiments

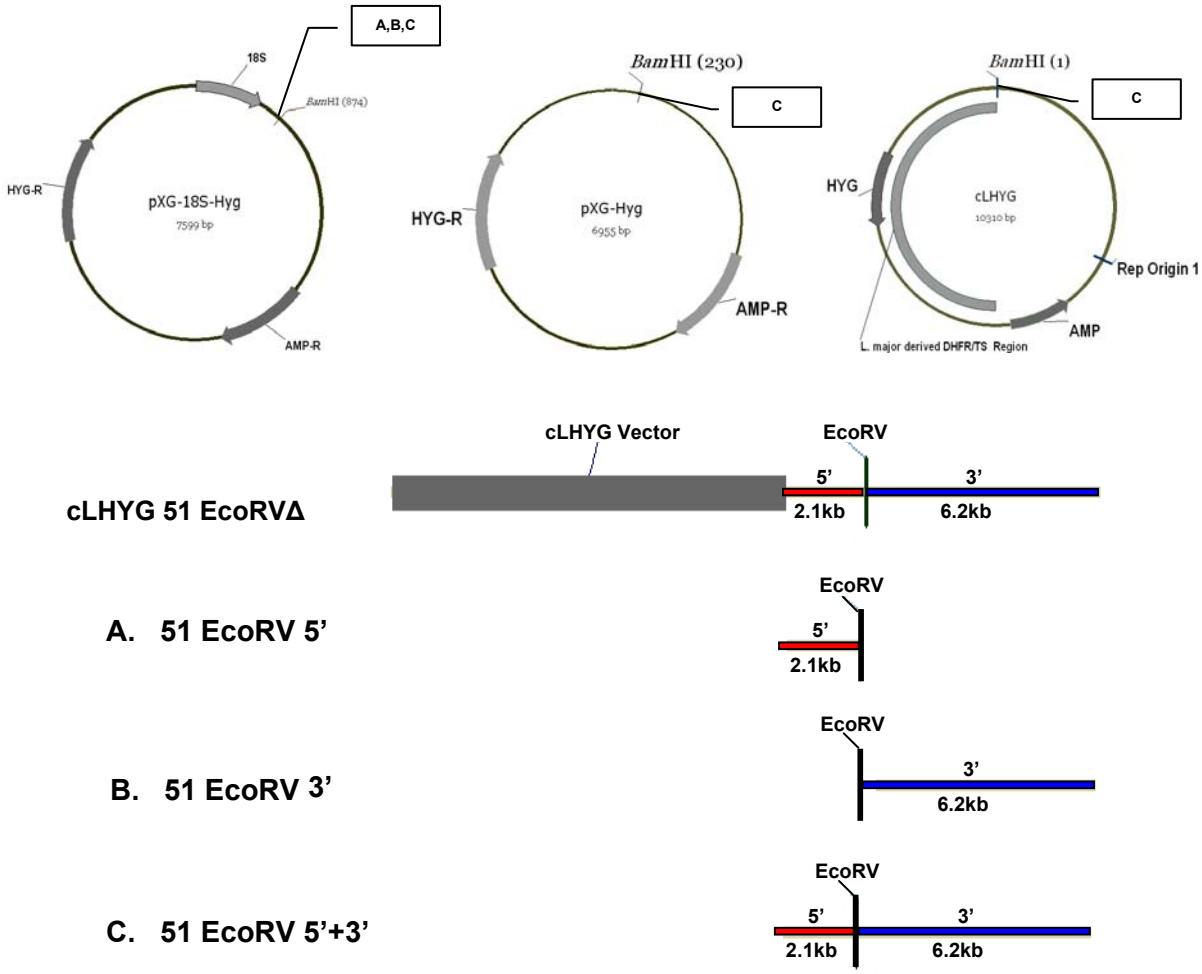


Figure 1. Cloning vectors and isolated regions of cosmid 51 insert
Three modified recipient vector constructs were generated (upper panel).Regions of the cosmid 51 EcoRVΔ insert were amplified by PCR and cloned into pXG-18S-Hyg (A) The 2.1kb region (from the vector to the internal *Eco*RV site) (B) The 6.2kb region (from the internal *Eco*RV site to the vector). (C) The 8.3kb regions (A, B) were ligated at the *Eco*RV internal site and cloned into all three recipient vectors.

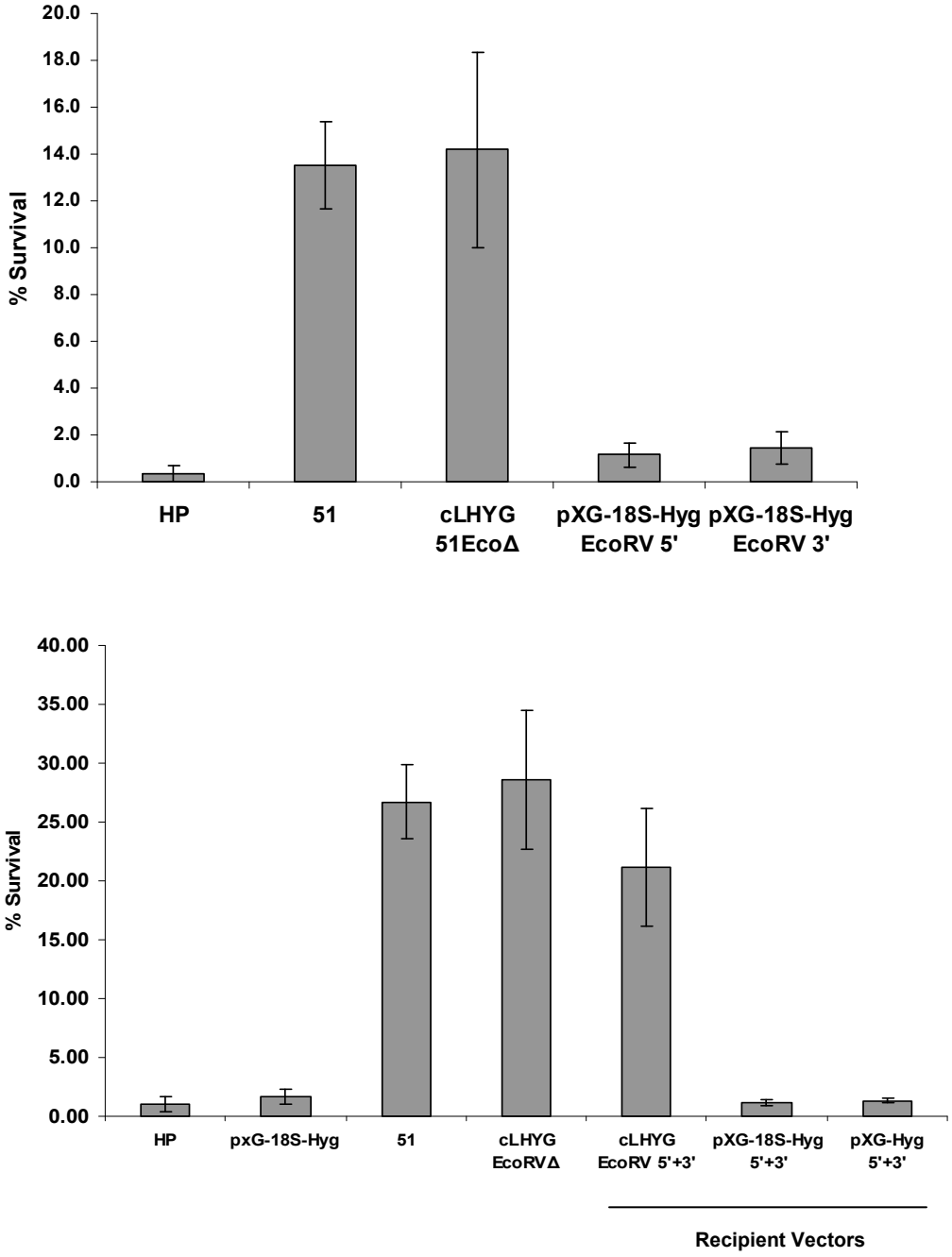


Figure 2. CML-resistance of 51 EcoRVΔ regions in cLHYG versus pXG-vector high passage transformants. Sensitivity of promastigotes incubated in 12.5% serum to CML was assayed. Stationary phase promastigotes included non-transfected high passage (HP), pXG-18S-Hyg transfected high passage control, cosmid 51 transfected high passage (51), cosmid 51 EcoRVΔ transfected high passage (cLHYG 51EcoRVΔ), and high passage cells transfected with pXG-18S-Hyg containing either (i) 2.1kb EcoRV 5' or the (ii)6.2kb EcoRV 3' (top panel) or high passage cells transfected with EcoRV 5'+3' inserts in one of three vectors; cLHYG, pXG-18S-Hyg and pXG-Hyg (bottom panel).

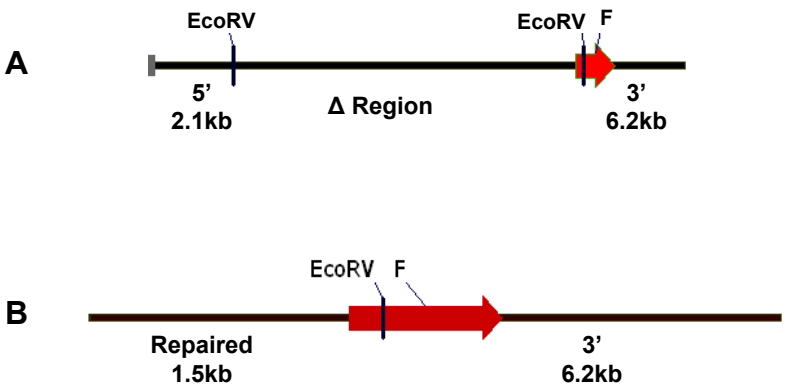


Figure 3. Repair of the EcoRVΔ region containing the predicted gene 51 F
(A) An illustration of the 519bp region of predicted gene 51 F that is eliminated in the ligation of 5' and 3' *Eco*RV restriction sites. (B) An illustration of the restored coding region of predicted gene F, generated for further characterization.

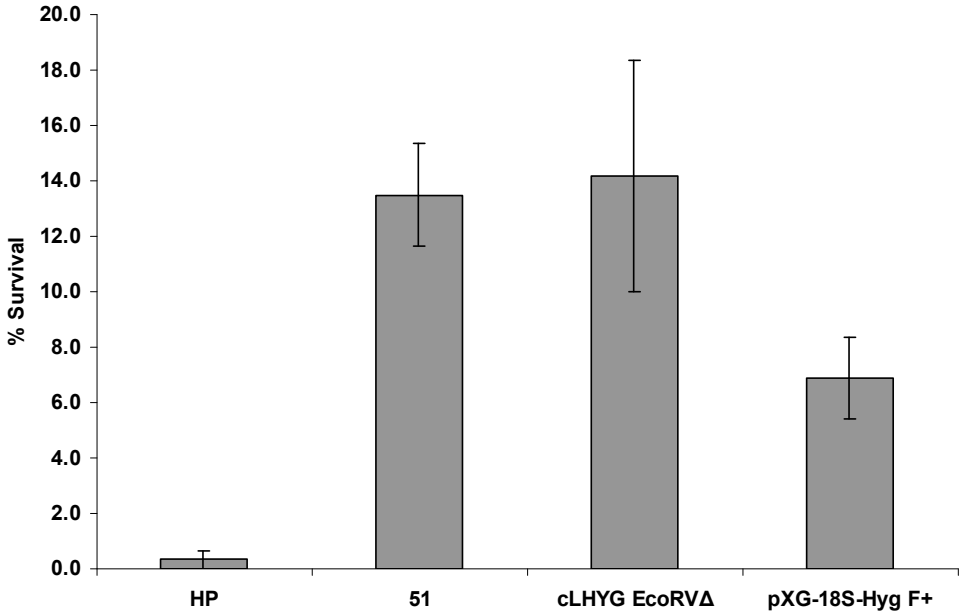


Figure 4. CML resistance of high passage transfectants containing gene 51F
Sensitivity of promastigotes incubated in 12.5% serum to CML was assayed. Stationary phase promastigotes included non-transfected high passage (HP), cosmid 51 transfected high passage (51), cosmid 51 EcoRVΔ transfected high passage (cLHYG 51EcoRVΔ), and high passage cells transfected with pXG-18S-Hyg plasmid containing the full PCR amplified coding region of 51F.

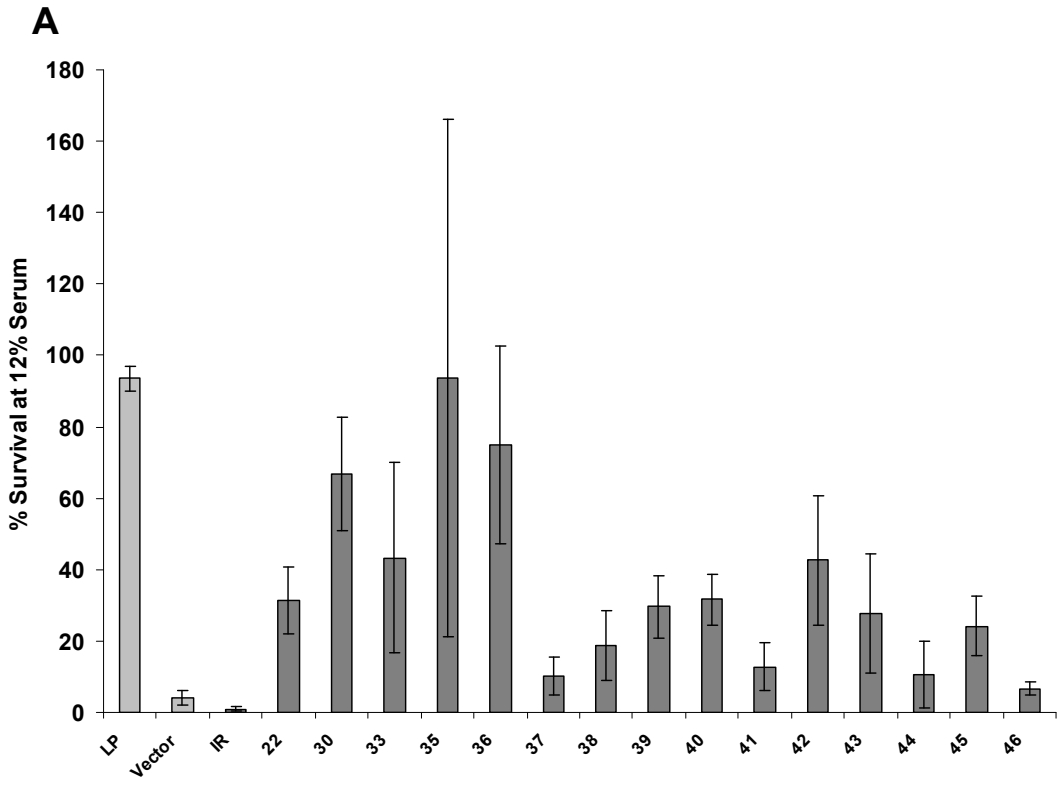


Figure 5A. Initial assays of cosmid transfected, serum selected high passage cells show variable levels of resistance to complement mediated lysis (CML-R). Clonally isolated, HP transformants were assayed for CML-R following expansion in liquid culture. Panel A represents complement assay results to identify serum resistant clones in stationary growth phase cultures. Controls included non-transfected, low passage (LP) promastigotes, HP cells transfected with cLHYG vector without insert (vector) and HP cells transfected with an irrelevant cosmid (IR). HP cosmid transformants had variable levels of CML-R in 12% pooled human serum. The mean survival of transformants was 42% with bars indicating the standard error of 2 to 5 experiments.

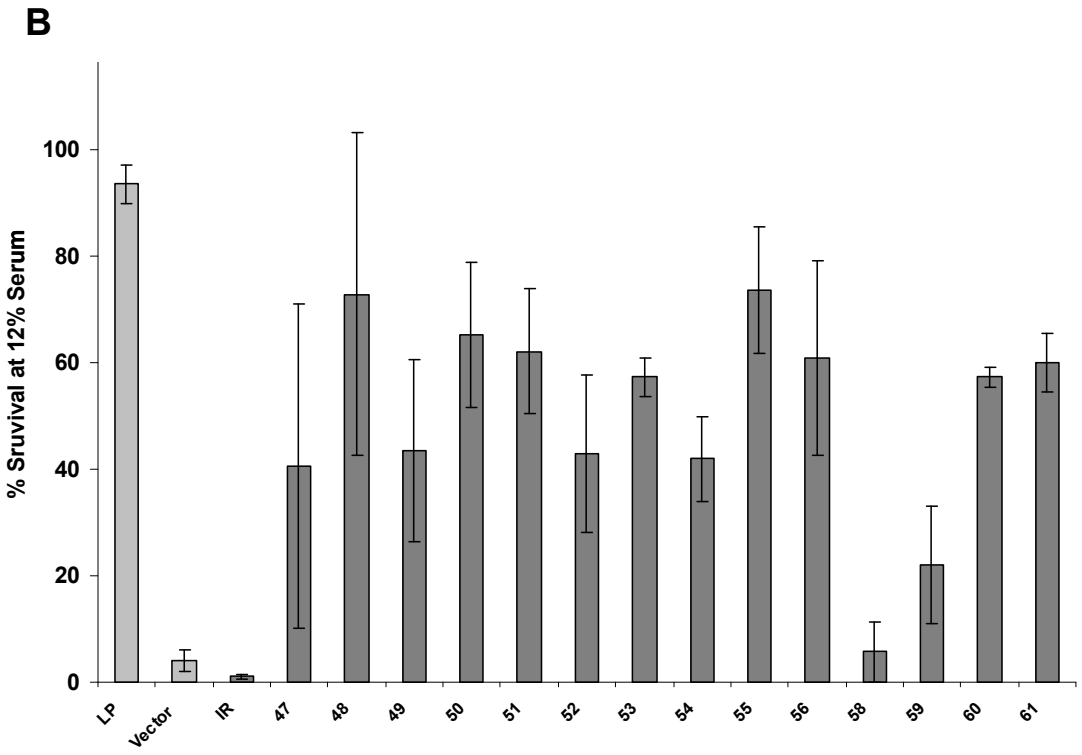


Figure 5B. Initial assays of cosmid transfected, serum selected high passage cells show variable levels of resistance to complement mediated lysis (CML-R). Clonally isolated, HP transformants were assayed for CML-R following expansion in liquid culture. Panel B represents complement assay results to identify serum resistant clones in stationary growth phase cultures. Controls included non-transfected, low passage (LP) promastigotes, HP cells transfected with cLHYG vector without insert (vector) and HP cells transfected with an irrelevant cosmid (IR). HP cosmid transformants had variable levels of CML-R in 12% pooled human serum. The mean survival of transformants was 42% with bars indicating the standard error of 2 to 5 experiments.

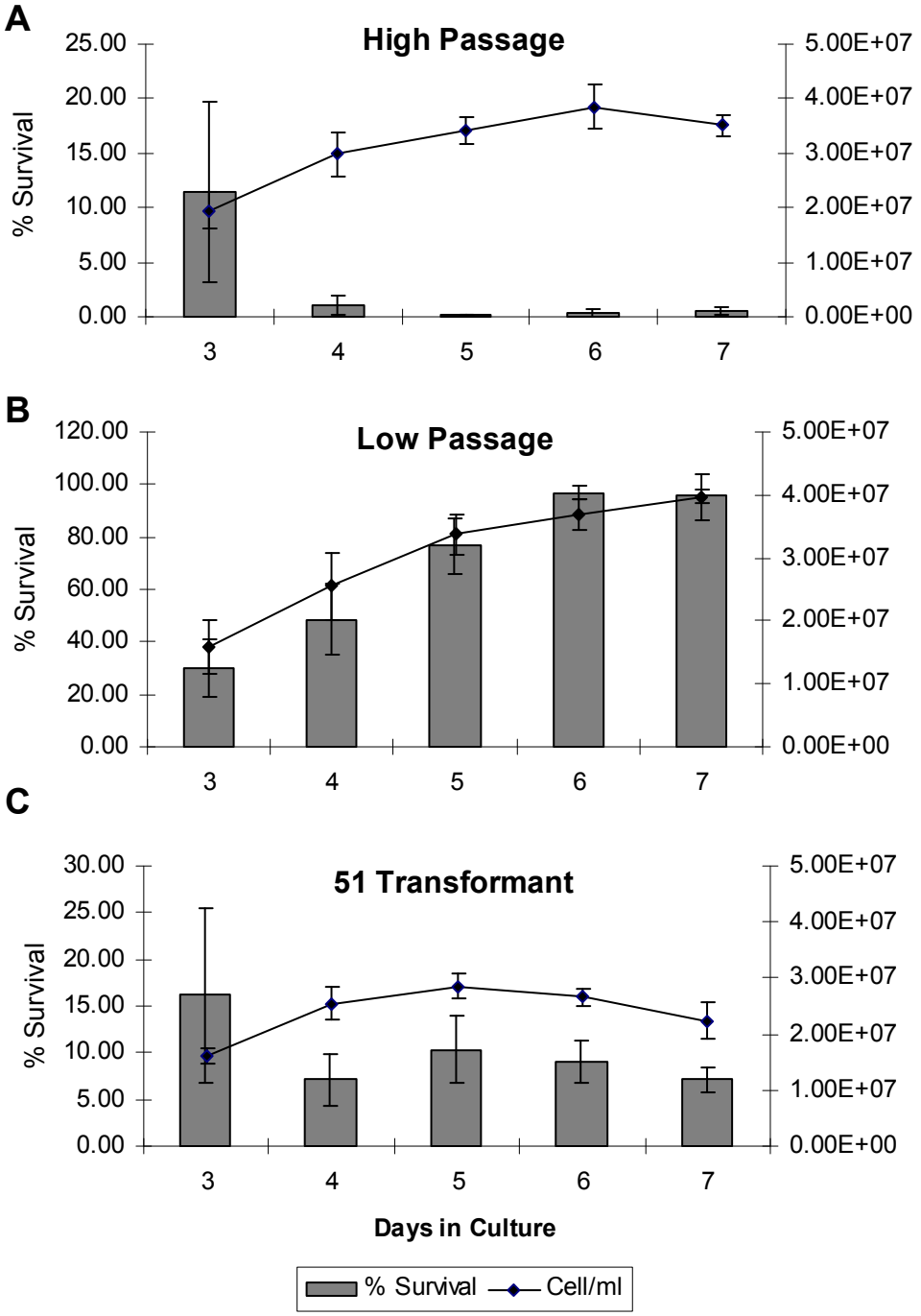


Figure 6 Complement assay time course experiments
Sensitivity of promastigotes incubated in 12.5% serum to cell lysis by complement (CML) was assayed. Promastigotes were grown in culture for 7 days. A growth curve was determined by on days 3-7 by daily quantification of cell density and CML-resistance levels were determined for (A) high passage, non-transfected (B) low passage, non-transfected and (C) high passage, cosmid 51 transfected cultures.

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